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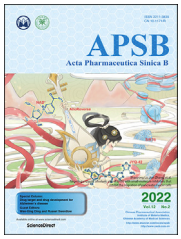

ORIGINAL ARTICLE

Modulating effects of RAMPs on signaling profiles of the glucagon receptor family

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Abstract Receptor activity-modulating proteins (RAMPs) are accessory molecules that form complexes with specific G protein-coupled receptors (GPCRs) and modulate their functions. It is established that RAMP interacts with the glucagon receptor family of GPCRs but the underlying mechanism is poorly understood. In this study, we used a bioluminescence resonance energy transfer (BRET) approach to comprehensively investigate such interactions. In conjunction with cAMP accumulation, $G\alpha_q$ activation and β-arrestin1/2 recruitment assays, we not only verified the GPCR−RAMP pairs previously reported, but also identified new patterns of GPCR−RAMP interaction. While RAMP1 was able to modify the three signaling events elicited by both glucagon receptor (GCGR) and glucagon-like peptide-1 receptor (GLP-1R), and RAMP2 mainly affected β-arrestin1/2 recruitment by GCGR, GLP-1R and glucagon-like peptide-2 receptor, RAMP3 showed a widespread negative impact on all the family

Abbreviations: AMY, amylin; B_{max}, maximum measured BRET value; BRET, bioluminescence resonance energy transfer; β_2-AR, β_2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; CGRP, calcitonin gene-related peptide; CLR, calcitonin-like receptor; EC_{50}, half maximal effective concentration; ECD, extracellular domain; E_{max}, maximal response; GCGR, glucagon receptor; GHRHR, hormone-releasing hormone receptor; GIPR, gastric inhibitory polypeptide receptor or glucose-dependent insulinotropic polypeptide; GLP-1R, glucagon-like peptide-1 receptor; GLP-2R, glucagon-like peptide-2 receptor; GPCRs, G protein-coupled receptors; pEC_{50}, negative logarithm of EC_{50}; RAMP, receptor activity-modulating protein; Rluc, *Renilla* luciferase; SBA, suspension bead array; SCTR, secretin receptor; SV, splice variant; TMD, transmembrane domain; VPAC2R, vasoactive intestinal polypeptide 2 receptor.

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Ligand selectivity

members except for growth hormone-releasing hormone receptor covering the three pathways. Our results suggest that RAMP modulates both G protein dependent and independent signal transduction among the glucagon receptor family members in a receptor-specific manner. Mapping such interactions provides new insights into the role of RAMP in ligand recognition and receptor activation.

1. Introduction

Receptor activity-modulating proteins (RAMPs) are a family of single transmembrane protein represented by three subtypes, RAMP1, 2 and 3. It has a conserved transmembrane domain, one large extracellular N terminus (~150 amino acids) and a short intracellular C terminus (~9 amino acids)[1]. RAMP was first found to bind with calcitonin-like receptor (CLR) and is required for CLR translocation from the endoplasmic reticulum to the cell membrane. Combination of CLR with RAMP1 forms the calcitonin gene-related peptide (CGRP) receptor complex, and that with RAMP2 and RAMP3 constitutes adrenomedullin receptors 1 and 2, respectively[2,3]. RAMP, as a member of accessory proteins, interacts with G protein-coupled receptor (GPCR) and displays receptor-dependent functional modulation of trafficking, selectivity and signaling[4]. For instance, association of the calcitonin receptor with RAMPs 1−3 yields three amylin (AMY) receptors: AMY1R, AMY2R and AMY3R, respectively[5,6]. Using different methods that monitor receptor trafficking subsequently identified interactions between other GPCRs and RAMPs, including glucagon receptor (GCGR), parathyroid hormone 1 and 2 receptors, vasoactive intestinal polypeptide 1 and 2 receptors (VPAC2R), corticotropin-releasing factor receptor 1 and secretin receptor (SCTR)[7−9]. Recently established suspension bead array immunoassay (SBA) for protein−protein interaction significantly expanded GPCR−RAMP pairs to cover all of the class B1 receptors[10,11].

The glucagon receptor family includes GCGR, growth hormone-releasing hormone receptor (GHRHR), gastric inhibitory polypeptide receptor (GIPR), glucagon-like peptide-1 and -2 receptors (GLP-1R and GLP-2R) and secretin receptor (SCTR)[12]. They regulate a variety of physiological functions in response to external stimuli *via* classical signaling pathways such as $G\alpha_s$-mediated cyclic adenosine monophosphate (cAMP) accumulation, $G\alpha_q$-mediated intracellular Ca^{2+} mobilization and G protein-independent β-arrestin1/2 recruitment[12−14].

Studies of GPCR−RAMP interactions will deepen our knowledge of receptor pharmacology that is valuable for the design of better therapeutics[15,16]. Thus, we investigated the effects of RAMP on signaling profiles (cAMP accumulation, $G\alpha_q$ activation and β-arrestin1/2 recruitment) mediated by the six members of this receptor family upon stimulation by endogenous ligands (glucagon, oxyntomodulin, GHRH, GIP, GLP-1, GLP-2 and secretin)[14]. A bioluminescence resonance energy transfer (BRET) assay was employed, instead of SBA[10,11] described in the literature, to measure the GPCR−RAMP interaction. Besides, SV1, a splice variant (SV) of GHRHR[17], was examined for comparison with the full-length receptor. Our results demonstrate that the association of the glucagon receptor family and RAMPs are broad and RAMPs specifically modulate their signaling profiles except for GHRHR.

2. Material and methods

2.1. Constructs

Full-length cDNA of the human CLR, vectors of the human RAMP1 and RAMP2, vectors of the human FLAG-RAMP1 and FLAG-RAMP2, as well as plasmids for the BRET assay were provided by Dr. Patrick M. Sexton. Plasmids used in the NanoBiT assay were gifts from Dr. Asuka Inoue. Full-length cDNA of the human RAMP3 was obtained from BGI (Beijing, China). Addition of FLAG- and HA-tags to receptors or RAMP3 was carried out by site-directed mutagenesis. All receptors were cloned to pcDNA3.1 vector and to the backbone of *Renilla* luciferase 8 (Rluc8) at the C terminus. RAMPs 1, 2 and 3 were ligated into both Ypet-N1 vector and pcDNA3 expression plasmid, respectively. All constructs were confirmed by DNA sequencing (GENEWIZ, Suzhou, China).

2.2. Cell culture

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 mmol/L sodium pyruvate (Gibco). HEK293A cell line was provided by Dr. Patrick M. Sexton and maintained in DMEM supplemented with 10% fetal bovine serum and nonessential amino acids (Gibco). All cell lines were incubated in a humidified environment at 37 °C in 5% CO_2.

For transient transfection, cells were seeded in either 6-well or 96-well plates. After 24 h incubation, cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in a 2:5 (mol/v) DNA:lipo2000 ratio. Following 24 h culturing, the transfected cells were ready for use.

2.3. BRET assay

To assess GPCR−RAMP interaction, receptor-Rluc8 was transfected at a constant concentration with increasing ratio of RAMP-Ypet from 1:0.25 to 1:16 to COS-7 cells. After transfection, cells were washed and incubated with 80 μL BRET buffer [calcium and magnesium free HBSS buffer (Gibco) supplemented with 10 mmol/L HEPES (Gibco) and 0.1% bovine serum albumin (BSA; Abcone, Shanghai, China), pH 7.4] in a 37 °C incubator for 30 min. Then Rluc8 specific substrate, coelenterazine H (Yeasen Biotech, Shanghai, China), was added to the incubation system with a final concentration of 5 μmol/L. The plates were read after 5 min using an EnVision multilabel instrument (PerkinElmer, Waltham, MA, USA). For titration assay, the signal ratio at 535 nm/470 nm was normalized and the curves were fitted for simple linear regression *vs*. one-site binding.

For β-arrestin1/2 recruitment, COS-7 cells were transfected with a receptor-Rluc8:RAMP:Venus-β-arrestin1/2 ratio of 1:1:2.

The next day, cells were washed and incubated with 80 μL BRET buffer. Then 10 μL coelenterazine H was added to each well with low light. Measurement was started 5 min thereafter. The initial 15-cycle reads were normalized as baseline and signals of ligand-induced responses were recorded for a total of 70 cycles.

2.4. *Immunofluorescence staining*

HEK293A cells were seeded in 6-well plates and transfected with 4 μg plasmid containing GPCR-HA and FLAG-RAMP at a ratio of 1:1. After 24 h, cells were collected and reseeded in 96-well plates until the cells reached 50%−70% confluences. They were washed with PBS before fixation with 4% paraformaldehyde for 15 min. Then they were washed three more times and blocked with 5% BSA plus 0.1% Triton X-100 for 1 h at room temperature (RT). Rabbit anti-HA primary antibody (Cell Signaling Technology, Danvers, MA, USA; 1:500) and mouse anti-FLAG primary antibody (Sigma−Aldrich, St. Louis, MO, USA; 1:300) were diluted with incubation buffer (PBS supplemented with 5% BSA) for 1 h followed by 3-time wash. Cells were reacted with 100 μL interaction buffer containing donkey anti-rabbit Alexa 488-conjugated secondary antibody and donkey anti-mouse Alexa 647-conjugated secondary antibody (Invitrogen; diluted 1:1000) at RT for 1 h in the dark. After final washing, nuclei were stained with Hoechst 33,258 for 5 min. Cells were imaged using high-resolution microscope DeltaVision™ Ultra (GE Healthcare, Boston, USA).

2.5. *cAMP accumulation*

COS-7 cells were seeded in 6-well plates and transfected with 4 μg plasmid containing receptor and RAMP at a ratio of 1:1. After 24 h, cells were collected and reseeded in 384-well plates at a density of 3000 cells/well. Following overnight incubation, cells were washed and incubated with stimulation buffer (calcium and magnesium free HBSS buffer, 5 mmol/L HEPES, 0.1% BSA, 0.5 mmol/L 3-isobutylene-1-methylxanthine). They were then treated with different concentrations of endogenous ligands for 40 min at RT. Detection reagent mixture was added to each well followed by 1 h additional incubation. The plates were read according to the LANCE protocol using an EnVision multilabel reader with the emission window ratio of 665 nm over 620 nm.

2.6. *$G\alpha_q$ activation*

HEK293A cells were seeded in 6-well plates and transfected with a plasmid mixture containing receptor:RAMP:$G\alpha_q$-LgBiT:$G\beta$:SmBiT-$G\gamma$:Ric-8A at a ratio of 1:1:1:3:3:4. Cells were transferred to 96-well plates at a density of 50,000 cells/well the next day. After 24 h, they were washed and incubated with 80 μL NanoBiT buffer (calcium and magnesium free HBSS buffer, supplemented with 10 mmol/L HEPES and 0.1% BSA, pH 7.4) in 37 °C for 30 min. Then 10 μL coelenterazine H was added to each well at a working concentration of 5 μmol/L followed by 2 h incubation. The plates were read at 30 s interval for 4 min as baseline, then read for 10 min after addition of ligand.

2.7. *Data analysis*

Data were analyzed using Prism 8 (GraphPad, San Diego, CA, USA). For signaling assays, data of individual experiments were normalized to the maximum responses in cells expressing only the receptor. Non-linear curve fit was performed using a three-parameter logistic equation [log (agonist *vs*. response)]. For time−course kinetic traces, the BRET ratio or luciferase value was corrected with post-stimulation basal reading and then normalized to the kinetic traces of each receptor−RAMP pair in the absence of ligand. All data are presented as mean ± standard error of mean (SEM). Significant differences were determined by one-way ANOVA with Dunnett's test.

3. Results

3.1. *GPCR−RAMP interaction*

A BRET assay was used to identify the association patterns between the glucagon receptor family members and RAMPs. This technique has been widely applied to study protein−protein interaction through detecting Rluc8 and Ypet attached to individual protein C terminus. We transfected a constant amount of receptor-Rluc8 with an increasing ratio of RAMP-Ypet into COS-7 cells and measured the signal ratio at 535 nm/470 nm. β_2-Adrenergic receptor (β_2-AR) was employed as negative control to set up the threshold[18]. CLR, a typical RAMP-interacting GPCR, was utilized as positive control[1]. All interaction curves reflect the best fit of a comparison between linear and hyperbolic fittings (Fig. 1). The fitting result inclined to the linear relationship was considered negative. For β_2-AR, the interaction with RAMPs 1 and 2 showed higher linear fitting which was hyperbolic in the case of RAMP3. Previous reports demonstrated that RAMP3 could translocate to the cell membrane when expressed alone. Higher surface expression of RAMP3 provided more random interactions with membrane receptors than that of RAMP1 and RAMP2, thereby deviating the fitting from linearity[7,18,19]. Therefore, we set up a threshold of $B_{max} > 0.35$ for RAMP3 interaction: below this value would be regarded negative. Of the protein pairs investigated, GLP-2R and RAMP1 exhibited a poor interaction compared to other pairs (Fig. 1A). It was noted that the interaction ability was negatively correlated with the value of $BRET_{50}$ (Table 1) which can be rank-ordered as weak ($BRET_{50}>5$), normal ($1<BRET_{50}<5$) and strong ($BRET_{50}<1$). GLP-1R/RAMP1, GLP-2R/RAMP2 and SV1/RAMP3 displayed weak interactions while that of GHRHR/RAMP2 and GIPR/RAMP3 were strong. This observation is supported by subsequent immunostaining studies showing the co-localization of members of the glucagon receptor family and RAMPs (Fig. 2). Quantitative analysis of RAMP surface expression suggests that co-transfection of CLR with RAMPs significantly improved the presence of the latter on the cell membrane (Supporting Information Fig. S6). For RAMP1, only co-expression with SCTR promoted cell surface translocation (Fig. S6A). The effect on RAMP2 co-expression is negligible whereas that of GCGR, SV1 and SCTR on RAMP3 expression was negative (Fig. S6B and S6C).

3.2. *cAMP accumulation*

The glucagon receptor family preferentially activates $G\alpha_s$ which subsequently activates adenylyl cyclase to generate cAMP[14]. To investigate the effect of RAMPs on this parameter, we transiently co-transfected individual receptors and each of the RAMP subtypes to COS-7 cells. As shown in Fig. 3 and Table 2, RAMP3 reduced GIP-elicited cAMP response by ~40-fold while the E_{max} value remained the same (Fig. 3H). It also decreased GLP-1 and

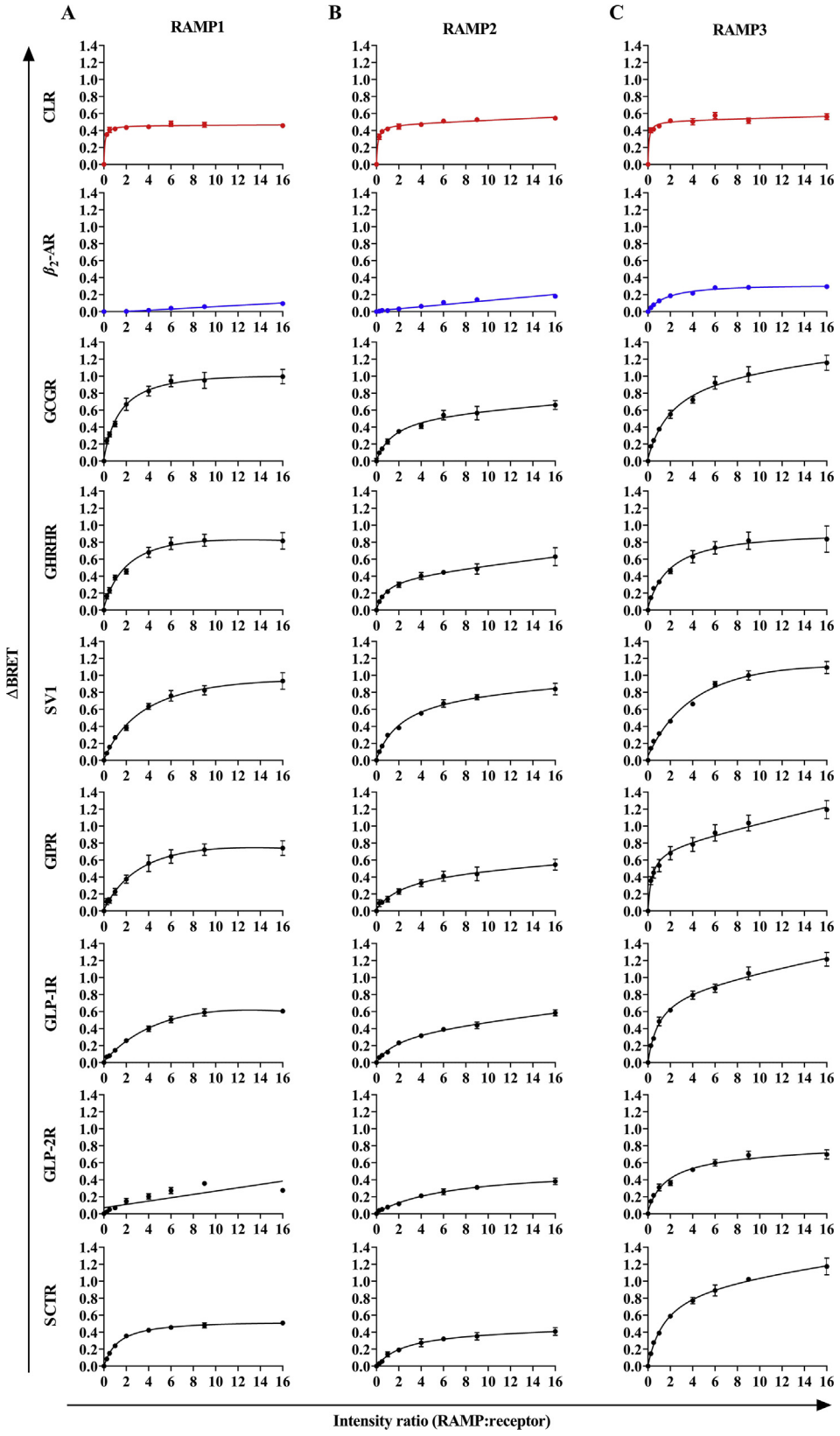


Figure 1 Screening of the interaction between the glucagon receptor family members and RAMPs. ΔBRET for each receptor−RAMP pair was observed in COS-7 cells. Curves were plotted as the level of increased RAMP:receptor signal ratio and calculated using the best-fit comparison for linearity *vs*. hyperbolic curve fitting (nonlinear fit of one site-binding). Curves are representative for each interaction between receptor and RAMP1 (A), RAMP2 (B) and RAMP3 (C), respectively. CLR was served as positive control (red) and β_2-AR was the negative control (blue). Average B_{max} and $BRET_{50}$ (K_d) values are provided in Table 1. Data shown are mean ± SEM from at least four independent experiments.

Table 1 Interaction of GPCR−RAMP pairs.

Receptor	RAMP1		RAMP2		RAMP3	
	B_{max}	$BRET_{50}$	B_{max}	$BRET_{50}$	B_{max}	$BRET_{50}$
CLR	0.460 ± 0.017	0.077 ± 0.016	0.468 ± 0.042	0.114 ± 0.019	0.511 ± 0.058	0.087 ± 0.037
β_2-AR	Linear fit	Linear fit	Linear fit	Linear fit	<0.35	1.865 ± 0.562
GCGR	1.149 ± 0.109	1.551 ± 0.362	0.578 ± 0.072	1.654 ± 0.496	1.102 ± 0.165	2.340 ± 0.716
GHRHR	1.122 ± 0.193	2.426 ± 0.837	0.393 ± 0.025	0.893 ± 0.165	0.948 ± 0.139	2.090 ± 0.661
SV1	1.364 ± 0.244	4.398 ± 1.155	0.843 ± 0.102	2.356 ± 0.581	1.842 ± 0.754	5.979 ± 3.064
GIPR	1.316 ± 0.203	4.600 ± 1.016	0.474 ± 0.122	2.751 ± 1.331	0.729 ± 0.061	0.352 ± 0.095
GLP-1R	2.039 ± 1.234	10.898 ± 6.262	0.397 ± 0.092	2.232 ± 1.079	0.862 ± 0.100	1.003 ± 0.333
GLP-2R	Linear fit	Linear fit	0.567 ± 0.234	7.451 ± 3.462	0.689 ± 0.128	1.655 ± 0.737
SCTR	0.573 ± 0.021	1.297 ± 0.128	0.434 ± 0.051	2.407 ± 0.572	0.995 ± 0.058	1.631 ± 0.232

All values are mean ± SEM of at least three independent experiments. Data were calculated using the best-fit comparison for linearity *vs.* hyperbolic curve fitting (nonlinear fit of one site-binding).
B_{max}, the maximum measured BRET value.
$BRET_{50}$ (K_d), the intensity ratio of RAMP:receptor that gives a half of the maximum response.

oxyntomodulin induced cAMP accumulation (by <10-fold; Fig. 3I and J). While RAMP3 had no effect on GHRHR it exhibited a marked response in SV1 expressing cells (Fig. 3G). Both RAMP1 and RAMP2 either caused a slight reduction (Fig. 3I and J) or had no influence on the E_{max} or EC_{50} values for any receptor.

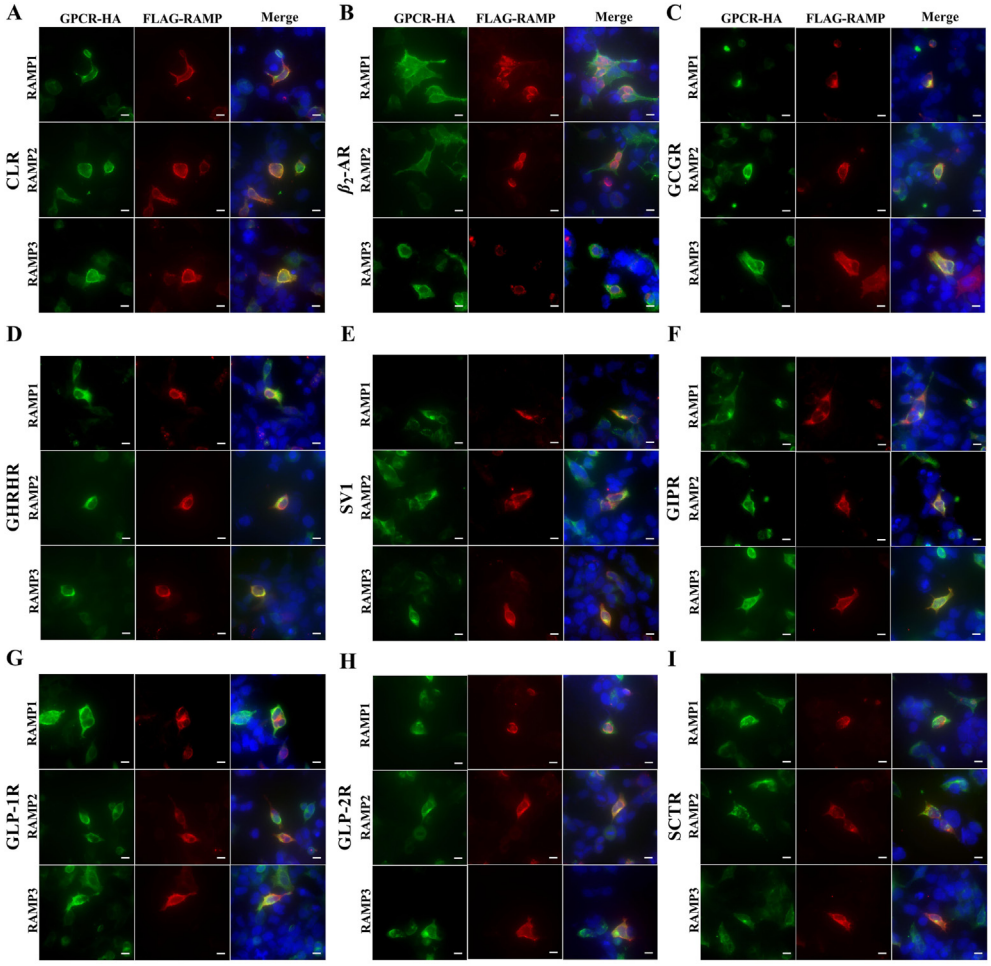


Figure 2 Co-localization of members of the glucagon receptor family and RAMPs. HEK293A cells were co-transfected with each FLAG-RAMP (red) and GPCR-HA (green) at a 1:1 ratio. After 24 h, each receptor−RAMP pair was stained with anti-HA and anti-FLAG monoclonal antibodies, respectively, using CLR (A) as positive and β_2-AR (B) as negative controls. Data shows representative results from three independent experiments at GCGR (C), GHRHR (D), SV1 (E), GIPR (F), GLP-1R (G), GLP-2R (H) and SCTR (I). Cells were observed by DeltaVision™ Ultra. Scale bar = 15 μm.

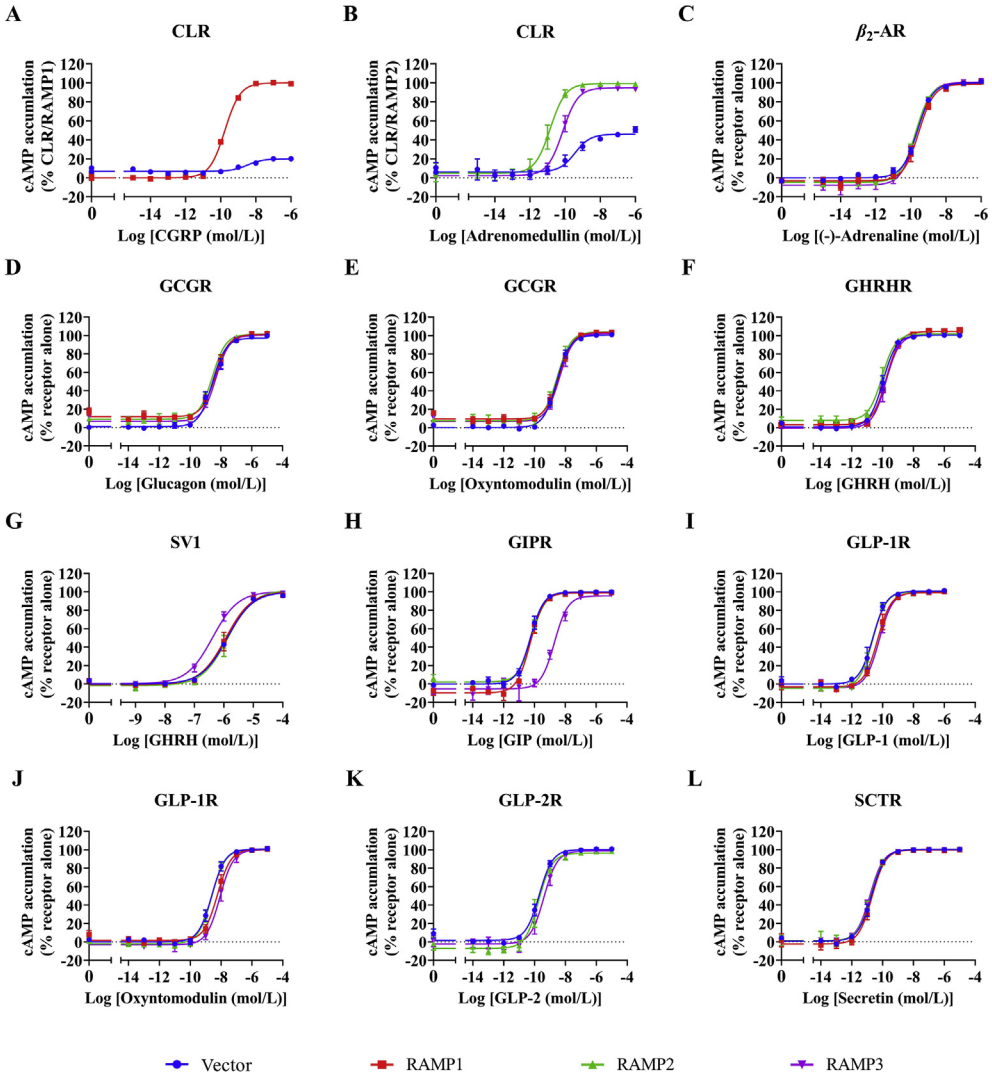


Figure 3 RAMP-mediated modulation of cAMP response. The glucagon receptor family members were transfected with either RAMP or vector to COS-7 cells. cAMP accumulation was elicited by endogenous ligand at CLR (A and B), β_2-AR (C), GCGR (D and E), GHRHR (F), SV1 (G), GIPR (H), GLP-1R (I and J), GLP-2R (K) and SCTR (L). CLR was served as positive control and β_2-AR was the negative control. Measurement of cAMP levels in the absence of RAMP at each receptor (CLR/RAMP1 or CLR/RAMP2 for response elicited by CGRP or adrenomedullin, respectively) was performed as a standard curve and then normalized to the maximal response for each RAMP interaction. Data were fitted with non-linear regression of three-parameter logistic curve. All values are mean ± SEM from at least three independent experiments.

3.3. *$G\alpha_q$ activation*

$G\alpha_q$ activation after ligand stimulation leads to activation of phospholipase C which cleaves phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol triphosphate related to Ca^{2+} mobilization[20]. A NanoBiT assay that fuses the large bit of nanoluciferase to the $G\alpha_q$-subunit and the small bit to the γ-subunit was used to assess the state of $G\alpha_q$[21] (Fig. 4, Supporting Information Fig. S1 and Table 2). For GCGR, both RAMPs 1 and 3 displayed reduced E_{max} values upon stimulation by glucagon and oxyntomodulin (Fig. 4D and E). The effects of RAMP1 on glucagon- and oxyntomodulin-induced maximal responses were weaker than that of RAMP3 (70.9 ± 7.3% *vs.* 42.1 ± 5.2% and 81.7 ± 10.8% *vs.* 24.5 ± 5%). For GHRHR, no RAMP was able to alter the concentration−response characteristics (Fig. 4F) whereas RAMP3 displayed a negative impact on the E_{max} stimulated by GLP-1 (63.7 ± 11%) or oxyntomodulin (75.5 ± 17.4%) (Fig. 4G and H). Similar phenomenon was observed with GLP-2R: 47.1 ± 6.8% of the E_{max} under the influence of RAMP3 (Fig. 4I). Besides, RAMP2 caused a nominal reduction in the potency of GLP-2R.

3.4. *β-Arrestin1/2 recruitment*

Independent of G protein signaling, β-arrestin recruitment is involved in receptor desensitization and endocytosis[22]. Arrestin 2 (also called β-arrestin1) and arrestin 3 (β-arrestin2) are widely expressed and bind to most GPCRs[23]. We used a BRET assay by attaching Rluc8 to receptor C terminus and Venus to β-arrestin1/2 N terminus (Fig. 5 and 6, Supporting Information Figs. S2 and S3). Both RAMPs 1 and 2 enhanced glucagon-mediated β-arrestin1 recruitment by GCGR (160.3 ± 14.7% and 185.5 ± 13%; Fig. 5D and Table 2). The negative impact of

Table 2 Modulation of signaling profiles of the glucagon receptor family of GPCRs.

Receptor	Ligand	Interaction	Assay							
			cAMP accumulation		$G\alpha_q$ activation		β-Arrestin1 recruitment		β-Arrestin2 recruitment	
			pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
CLR	CGRP	Vector	8.5 ± 0.2	20.0 ± 1.2	N.D.	N.D.	7.3 ± 1.8	16.9 ± 10.6	N.D.	N.D.
		RAMP1	9.8 ± 0	100 ± 0.8	7.2 ± 0.1	100 ± 3.7	6.9 ± 0.3	100 ± 8.6	6.8 ± 0.2	100 ± 7.6
	Adrenomedullin	Vector	9.4 ± 0.2	46.3 ± 2.9	N.D.	N.D.	N.A.	N.A.	N.A.	N.A.
		RAMP2	10.8 ± 0.1	100 ± 2.5	N.D.	N.D.	6.8 ± 0.5	100 ± 17.2	5.8 ± 0.4	100 ± 16.2
		RAMP3	10.1 ± 0.1	95.6 ± 1.7	N.D.	N.D.	6.7 ± 1.4	17.9 ± 11.7	6.6 ± 0.5	58.4 ± 10.9
β_2-AR	(−)-Adrenaline	Vector	9.6 ± 0	100 ± 1.3	6.9 ± 0.2	100 ± 6.7	6.2 ± 0.3	100 ± 11.5	6.4 ± 0.1	100 ± 4.1
		RAMP1	9.6 ± 0.1	98.9 ± 1.9	6.5 ± 0.2	102.3 ± 10.3	6 ± 0.4	77.3 ± 11.1	6.7 ± 0.2	79.1 ± 5.7
		RAMP2	9.7 ± 0	99.7 ± 1.5	6.3 ± 0.4	116.4 ± 20.5	5.8 ± 0.5	83.4 ± 15	6.5 ± 0.2	90 ± 5.4
		RAMP3	9.7 ± 0.1	100.7 ± 2	6.9 ± 0.3	98.7 ± 11.6	6 ± 0.4	104.6 ± 15.7	6.4 ± 0.2	79.2 ± 6.1
GCGR	Glucagon	Vector	8.5 ± 0.1	100 ± 1.9	6.8 ± 0.2	100 ± 8.1	5.5 ± 0.1	100 ± 6.3	5.1 ± 0.1	100 ± 4.6
		RAMP1	8.4 ± 0.1	104 ± 1.9	6.8 ± 0.2	70.9 ± 7.3*	5.3 ± 0.2	160.3 ± 14.7*	5.1 ± 0.2	76 ± 7.6
		RAMP2	8.6 ± 0.1	104.3 ± 2	6.7 ± 0.2	102.1 ± 7.6	5.0 ± 0.1	185.5 ± 13**	5.2 ± 0.3	110 ± 13.9
		RAMP3	8.3 ± 0.1	103.3 ± 1.9	7.3 ± 0.3	42.1 ± 5.2**	5.0 ± 0.4	107.9 ± 21.2	5.2 ± 0.2	78.4 ± 7.4
	Oxyntomodulin	Vector	8.6 ± 0	100 ± 1.2	7 ± 0.2	100 ± 9.4	N.D.	N.D.	N.D.	N.D.
		RAMP1	8.4 ± 0.1	103.1 ± 1.7	7 ± 0.3	81.7 ± 10.8	N.D.	N.D.	N.D.	N.D.
		RAMP2	8.5 ± 0.1	103.4 ± 2.5	7 ± 0.3	113 ± 13.6	N.D.	N.D.	N.D.	N.D.
		RAMP3	8.4 ± 0.1	101.7 ± 2.2	9.9 ± 0.4***	24.5 ± 5**	N.D.	N.D.	N.D.	N.D.
GHRHR	GHRH	Vector	10 ± 0	100 ± 1.2	10.7 ± 0.2	100 ± 6.9	5.3 ± 0.6	100 ± 25.3	N.D.	N.D.
		RAMP1	9.8 ± 0	104.1 ± 1.2	11.2 ± 0.3	91.8 ± 8.3	4.8 ± 0.6	123.5 ± 42.2	N.D.	N.D.
		RAMP2	10 ± 0.1	101.5 ± 1.4	10.5 ± 0.3	105.1 ± 9.4	4.7 ± 0.4	124 ± 34	N.D.	N.D.
		RAMP3	9.8 ± 0.1	100.3 ± 1.5	10.9 ± 0.3	87.9 ± 8	4.4 ± 0.6	101.9 ± 45.7	N.D.	N.D.
SV1	GHRH	Vector	5.9 ± 0	100 ± 1.8	N.A.	N.A.	5.4 ± 0.3	100 ± 11.2	N.D.	N.D.
		RAMP1	5.9 ± 0.1	100.3 ± 3.4	N.A.	N.A.	4.2 ± 0.3	188.4 ± 36.8	N.D.	N.D.
		RAMP2	5.9 ± 0.1	102 ± 4.2	N.A.	N.A.	4.1 ± 0.3	181.8 ± 44.2	N.D.	N.D.
		RAMP3	6.4 ± 0.1**	100.3 ± 2.0	N.A.	N.A.	4.7 ± 0.5	168.1 ± 49.1	N.D.	N.D.
GIPR	GIP	Vector	10.3 ± 0.1	100 ± 1.3	N.A.	N.A.	4.9 ± 0.3	100 ± 17.7	6.5 ± 0.4	100 ± 12.7
		RAMP1	10.3 ± 0.1	99 ± 1.8	N.A.	N.A.	5.5 ± 0.4	81.5 ± 16.9	5.6 ± 0.6	114 ± 29.6
		RAMP2	10.2 ± 0	99.3 ± 1.2	N.A.	N.A.	4.9 ± 0.3	82.1 ± 17.4	6.2 ± 0.3	103.5 ± 14.9
		RAMP3	8.7 ± 0.1***	95.7 ± 3.3	N.A.	N.A.	5.2 ± 0.4	61.2 ± 15.4	5.8 ± 0.4	130.4 ± 28.7
GLP-1R	GLP-1	Vector	10.6 ± 0.1	100 ± 1.8	11.8 ± 0.2	100 ± 5	6.7 ± 0.1	100 ± 3.8	6.8 ± 0.2	100 ± 6.1
		RAMP1	10.3 ± 0.1*	98.5 ± 1.7	11.6 ± 0.3	86.2 ± 5.6	6.6 ± 0.1	106.3 ± 4.7	6.9 ± 0.1	96 ± 4.7
		RAMP2	10.6 ± 0.1	99.4 ± 1.4	11.2 ± 0.2	83.8 ± 5.3	6.5 ± 0.2	105 ± 5.9	6.9 ± 0.1	104 ± 5.1
		RAMP3	10.2 ± 0.1**	98.8 ± 1.7	11 ± 0.5	63.7 ± 11*	6.4 ± 0.1	89.1 ± 2.8	6.3 ± 0.2	74 ± 6.9*
	Oxyntomodulin	Vector	8.6 ± 0.1	100 ± 1.6	10.8 ± 0.2	100 ± 5.4	5.8 ± 0.2	100 ± 12	5.9 ± 0.1	100 ± 5.2
		RAMP1	8.2 ± 0.1**	99.8 ± 2.1	10.2 ± 0.3	107.8 ± 8.4	5.9 ± 0.2	122.3 ± 11	5.9 ± 0.1	101 ± 5.8
		RAMP2	8.6 ± 0.1	99.6 ± 1.8	10.6 ± 0.4	102.7 ± 9.5	5.7 ± 0.2	119.9 ± 12	6.1 ± 0.1	125 ± 6.9*
		RAMP3	8.1 ± 0.1***	99.4 ± 2.5	9.9 ± 0.7	75.5 ± 17.4	5.4 ± 0.2	116 ± 14	5.7 ± 0.1	85 ± 6.3
GLP-2R	GLP-2	Vector	9.7 ± 0.1	100 ± 1.4	10.5 ± 0.3	100 ± 7.5	7.1 ± 0.2	100 ± 6	7.1 ± 0.1	100 ± 4.1
		RAMP2	9.7 ± 0.1	96.2 ± 2.7	9.2 ± 0.2	119.2 ± 9.9	7 ± 0.1	89.5 ± 4	7 ± 0.1	79.3 ± 2**
		RAMP3	9.4 ± 0.1	98.7 ± 2.7	11 ± 0.5	47.1 ± 6.8**	6.8 ± 0.2	77.9 ± 5*	6.7 ± 0.1*	75.6 ± 3.5**

(*continued on next page*)

Table 2 (*continued*)

Receptor	Ligand	Interaction	Assay							
			cAMP accumulation		$G\alpha_q$ activation		β-Arrestin1 recruitment		β-Arrestin2 recruitment	
			pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
SCTR	Secretin	Vector	10.7 ± 0	100 ± 1	7.7 ± 0.4	100 ± 14.1	7 ± 0.1	100 ± 5.6	7.2 ± 0.1	100 ± 4
		RAMP1	10.7 ± 0.1	99.6 ± 1.5	7.9 ± 0.3	127.7 ± 13.5	7 ± 0.1	91.8 ± 3.6	6.8 ± 0.2	81 ± 6.2
		RAMP2	10.8 ± 0.1	99.6 ± 1.7	8.2 ± 0.3	106.8 ± 12.5	7.1 ± 0.1	105.9 ± 6.4	7.1 ± 0.2	105 ± 6.8
		RAMP3	10.8 ± 0	99.4 ± 0.9	7.6 ± 0.6	97.8 ± 21.1	6.8 ± 0.2	69.1 ± 4.9**	6.9 ± 0.1	67 ± 4.2**

cAMP accumulation and β-arrestin1/2 recruitment assays were performed in COS-7 cells. $G\alpha_q$ activation assay was conducted in HEK293A cells. All the measures were fitted to non-linear regression three-parameter logistic curves. pEC_{50} is the negative logarithm of the concentration of an agonist that gives a half of the maximum response. E_{max} is the percentage (%) of the maximum response in cells expressing receptor only (CLR/RAMP1 or CLR/RAMP2 for response elicited by CGRP or adrenomedullin, respectively).
All values are mean ± SEM of at least three independent experiments. Statistical analysis was carried out by comparing the control responses in the absence of RAMP.
$^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, determined by one-way ANOVA with Dunnett's test.
N.A., no robust stimulation was detected at the highest concentration.
N.D., values that could not be determined without complete curve fit.

RAMP3 on the maximal β-arrestin1 responses at GLP-2R (77.9 ± 5%) and SCTR (69.1 ± 4.9%) were marginal (Fig. 5K and L). In the case of β-arrestin2 (Fig. 6 and Table 2), RAMP3 decreased the E_{max} value of GLP-1-mediated recruitment while RAMP2 increased oxyntomodulin-stimulated response at GLP-1R (Fig. 6I and J). Similar weakening impact was seen with RAMP2 (79.3 ± 2%) and RAMP3 (75.6 ± 3.5%) on the maximal responses at GLP-2R (Fig. 6K). For SCTR, RAMP3 exhibited a feeble decrease in maximal signaling (67 ± 4.2%, Fig. 6L).

3.5. *Receptor pharmacology*

Supporting Information Table S1 and Fig. 7 show that RAMP has an extensive interaction with the glucagon receptor family members albeit it is rather weak between RAMP1 and GLP-2R. Looking at the subtype specificity, RAMP1 exerts effects on GCGR and GLP-1R through cAMP production, $G\alpha_q$ activation and β-arrestin1 recruitment. It appears biased towards cAMP accumulation at GLP-1R compared to GCGR that trends towards $G\alpha_q$ activation. RAMP2 only participates in β-arrestin1/2 recruitment by GCGR, GLP-1R and GLP-2R. RAMP3 demonstrates a broad spectrum of negative modulation covering all three tested pathways involving GCGR, GIPR, GLP-1R, GLP-2R and SCTR.

Since RAMP3 has a negative impact on GLP-1-induced signaling, it may also affect receptor scavenging. Meanwhile, β-arrestin2 recruitment is increased upon stimulation by oxyntomodulin in the presence of RAMP2. Therefore, we performed ligand-induced internalization assay with GLP-1R in the presence or absence of RAMP2/3. It was found that RAMP did not affect the speed of GLP-1R internalization elicited by GLP-1, while RAMP2 caused a rapid loss of cell surface GLP-1R upon oxyntomodulin stimulation, suggesting that RAMP2 may promote receptor scavenging through β-arrestin recruitment (Supporting Information Fig. S7).

Except for GHRHR, other five family members seem to be functionally modulated by at least one RAMP. Oxyntomodulin is a dual-agonist of GCGR and GLP-1R[24,25]. For GLP-1R, RAMPs 1, 2 and 3 were shown to affect oxyntomodulin-induced cAMP and β-arrestin2 recruitment, while RAMPs 1 and 3, but not RAMP2, only took part in $G\alpha_q$ pathway at GCGR. It appears that RAMP is capable of modifying signaling profiles elicited by different ligands on the same receptor. RAMP3 reduced both glucagon- and oxyntomodulin-induced $G\alpha_q$ responses at GCGR. RAMP1 suppressed $G\alpha_q$ response but promoted β-arrestin1 recruitment which could also be enhanced by RAMP2 upon glucagon stimulation. For GLP-1R, RAMPs 1 and 3 caused a decreased cAMP signaling under the influence of either GLP-1 or oxyntomodulin, whereas RAMP2 specifically enhanced oxyntomodulin-stimulated β-arrestin2 recruitment and RAMP3 exerted a negative impact on GLP-1-induced $G\alpha_q$ activation and β-arrestin2 recruitment. While GIPR-mediated cAMP signaling was weakened by RAMP3, RAMPs 2 and 3 restrained β-arrestin2 recruitment and $G\alpha_q$ response at GLP-2R. The effects of RAMP3 on β-arrestin1/2 recruitment at both GLP-2R and SCTR were also negative. Although no modulating effect was seen at GHRHR, the impact of RAMP3 on cAMP accumulation at SV1 was noticeable (Fig. 3F and G).

4. Discussion

To investigate the role of RAMPs in modulating signaling pathways of the glucagon receptor family of GPCRs, we established a BRET assay to detect interactive pairs. Compared to the results

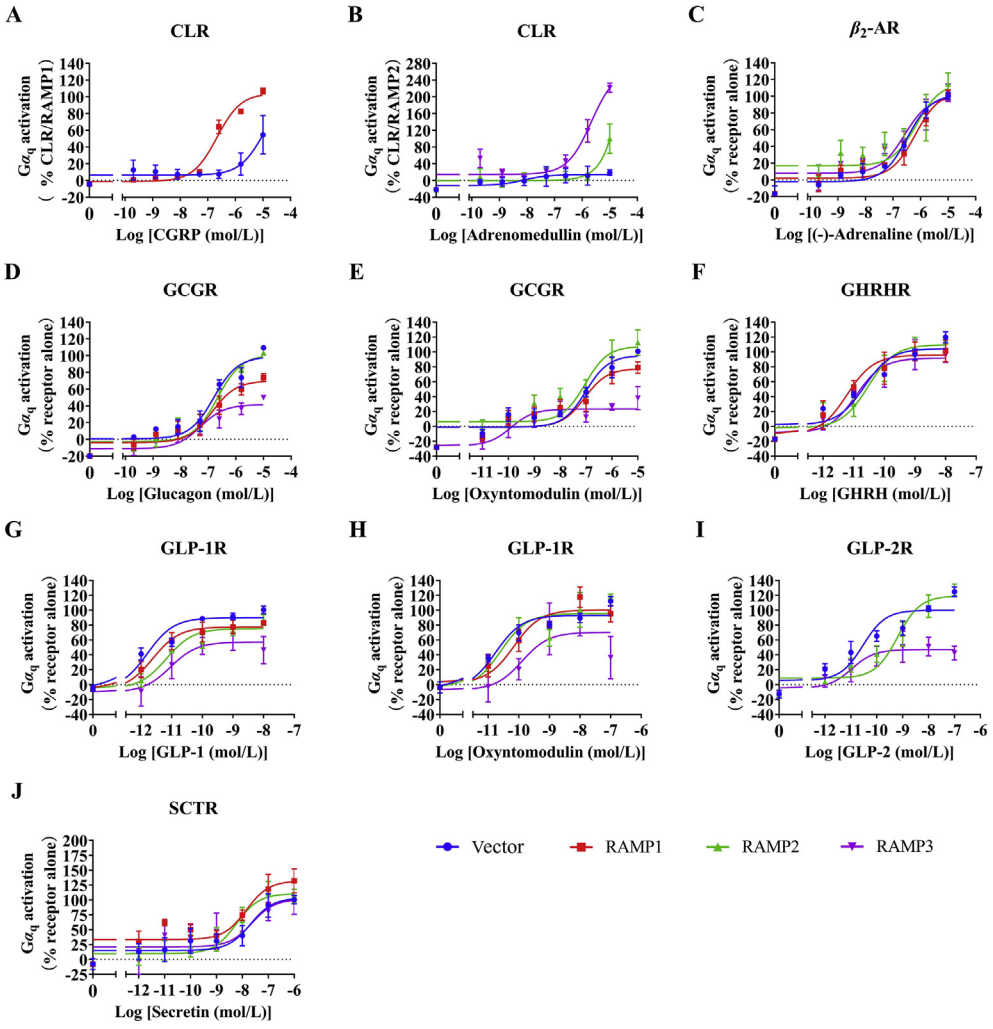


Figure 4 RAMP-mediated modulation of Gα_q activation. Measurement of Gα_q activation was made in HEK293A cells using a NanoBiT luciferase assay. Gα_q activation was elicited by endogenous ligands at CLR (A and B), β_2-AR (C), GCGR (D and E), GHRHR (F), GLP-1R (G and H), GLP-2R (I) and SCTR (J). CLR was served as positive control and β_2-AR was the negative control. For quantification of concentration−responses, area-under-the-curve data were analyzed after subtracting the post-stimulation baseline. Data were fitted to non-linear regression of three-parameter logistic curve and normalized to the maximal response in cells expressing receptor only (CLR/RAMP1 or CLR/RAMP2 for response elicited by CGRP or adrenomedullin, respectively). Values shown are mean ± SEM from at least three independent experiments.

generated by multiplexed SBA techniques, we confirmed most GPCR−RAMP interactions reported previously. However, the association between GHRHR and RAMP1 was readily observed in this study (Fig. 1A) but was claimed to be insignificant with the SBA assay[11], a phenomenon that was noted between GLP-2R and RAMP1 as well (Fig. 1A). Since HEK293T cells utilized in that study express endogenous RAMP1, it may cause non-specific binding to the receptor in question[4]. High background noise would also reduce protein−protein interaction signal. Such a discrepancy might have been resulted from failure to meet the protein pair distance (<10 nm) required for BRET[26,27]. Another difference between BRET and SBA is that the former is a quantitative and straightforward measurement in living cells. Although SV1 has a naturally truncated extracellular domain (ECD)[17,28], it still showed interactions with all three RAMPs. Studies of the interactome variability by comparing SV1 with GHRHR support the contribution of ECDs to GPCR−RAMP interface[29]. The published structures of CLR/RAMPs[30,31] and chimeric secretin-GLP-1R/RAMP3[9] indicate that the complex formation requires the association between RAMP and receptor transmembrane domain (TMD). It is likely the high conservation of TMDs among the glucagon receptor family members and the spatial similarity of RAMPs provide a constitutive basis for general interactions between GPCR−RAMP pairs[4,32,33].

Interestingly, the interaction between GLP-2R and RAMP1 is different from other pairs. To understand the distinct RAMP1-binding profiles between CLR and GLP-2R, we performed the sequence analysis and structural comparison of the RAMP1-binding sites based on the reported cryo-EM structures (CGRP-CLR−RAMP1−G_s complex: 6E3Y; GLP-2−GLP-2R−G_s complex: 7D68; Supporting Information Figs. S4 and S5)[31,34]. RAMP1 forms extensive hydrophobic interactions with TMs 3−5 as well as several polar contacts with ECL2[31]. However, by adopting unique amino acids that are different from other members in the glucagon receptor family in several positions, direct repulsion between GLP-2R TMD and RAMP1 was observed

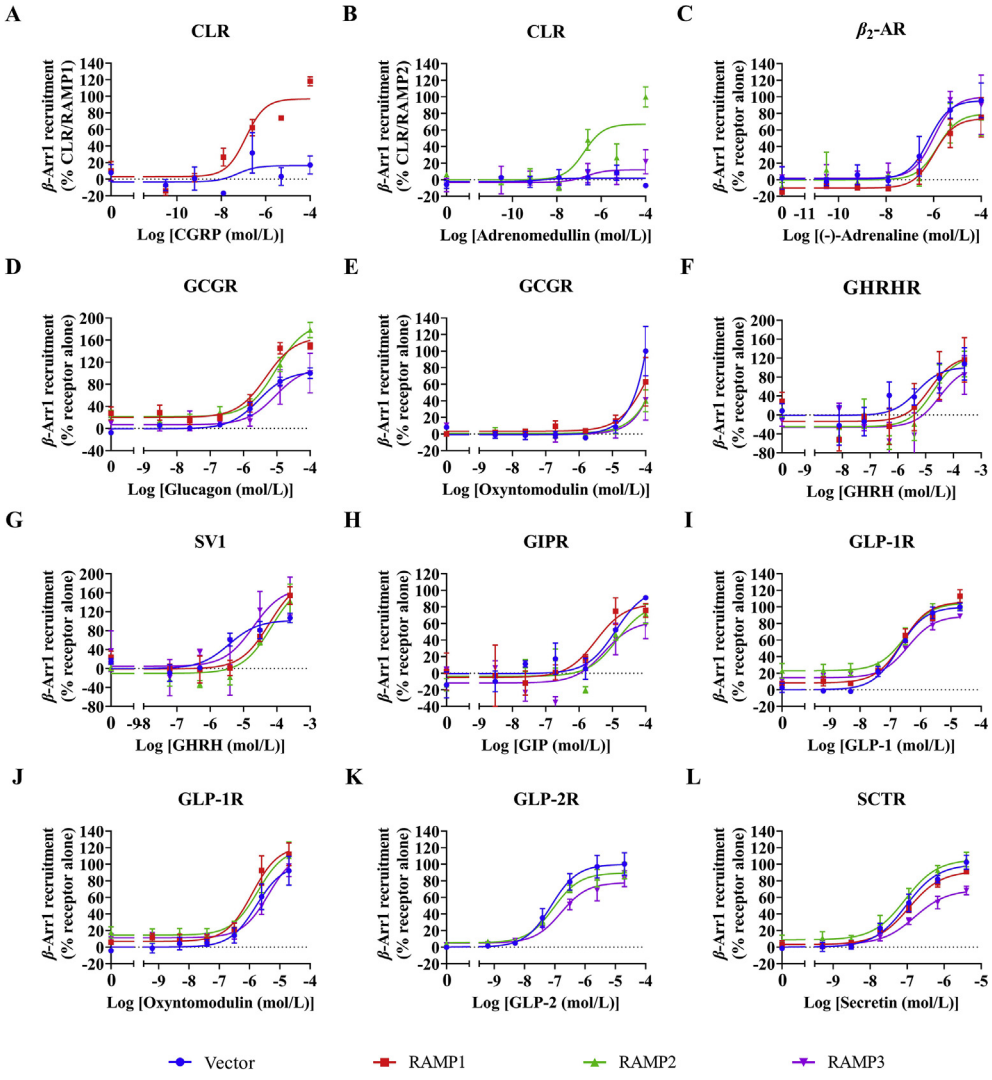


Figure 5 RAMP-mediated modulation of β-arrestin1 recruitment. β-Arrestin1 (β-Arr1) recruitment was assessed in COS-7 cells using a BRET assay. β-Arrestin1 level with each RAMP was normalized to the maximal response in the absence of RAMP at β_2-AR (C), GCGR (D and E), GHRHR (F), SV1 (G), GIPR (H), GLP-1R (I and J), GLP-2R (K) and SCTR (L). CLR was served as positive control and β_2-AR was the negative control. CLR was normalized to the measurement in the presence of RAMP1 (elicited by CGRP) and RAMP2 (elicited by adrenomedullin). Data shown are mean ± SEM from at least three independent experiments. Concentration−response curves were fitted to non-linear regression curves.

(Fig. S4). K337^{ECL2} and K338^{ECL2} in ECL2 of GLP-2R do not make polar interaction with RAMP1 while T288^{ECL2} and H289^{ECL2} (CLR) in the equivalent position form the RAMP1-ECL2 interface (Fig. S5B)[31,35]. Compared with the dense contacts between Q140 (RAMP1) and W254$^{4.44}$ (CLR), the large side-chain of R302$^{4.44}$ may push the C-terminal of RAMP1 away from the TMD (Fig. S5C). At the C-terminal of TM3 in GLP-2R, proline emerges several residues ahead compared to CLR and other members. It points to a shorter TM3 and conformational change in intracellular loop 2 which may impact the corresponding K142 (RAMP1)−V243^{ICL2} interaction (Fig. S5D). These special structural features of GLP-2R would hinder the binding of RAMP1 to GLP-2R.

The results of co-localization experiments (Fig. 2) are consistent with the above findings. However, compared to the BRET assay, analysis of RAMP cell surface expression indicates that only a few receptors exhibited an impact (Supporting Information Fig. S6). For instance, GCGR, SV1 and SCTR downregulated RAMP3 membrane expression, suggesting that cytoplasm remains its action site.

Three signaling events (cAMP generation, Gα_q activation and β-arrestin1/2 recruitment) were assessed to study the effects of RAMPs on six closely related class B1 receptors. Addition of either GIP or GHRH to cells transfected with respective receptor alone did not affect Gα_q response at GIPR or SV1 (data not shown). GIPR appears predominantly to signal *via* Gα_s rather than Gα_q[36] and Ca^{2+} mobilization[36,37], whereas the noticeable impact of RAMP3 on GHRH-induced cAMP accumulation at SV1 (Fig. 3G and H) supports the role of ECD in RAMP-modulated G protein activation[30].

Of interest is that RAMP showed robust association with some of the receptors but failed to affect their signaling pathways. Since RAMP is involved in receptor trafficking, recycling and degradation[9,18,38], pharmacological effects observed in the present study may reflect the dominant action out of its multifaceted functionalities which sometimes relies on the cell line background.

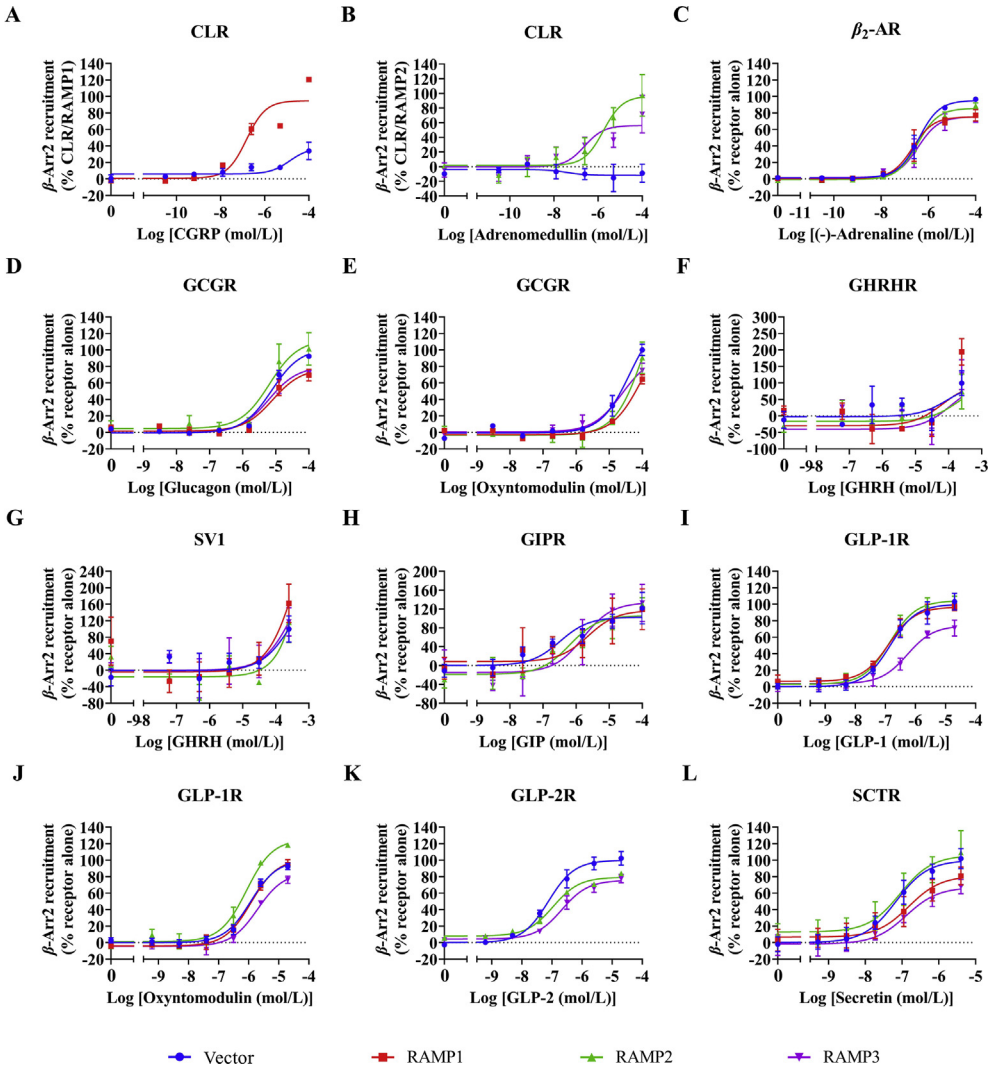
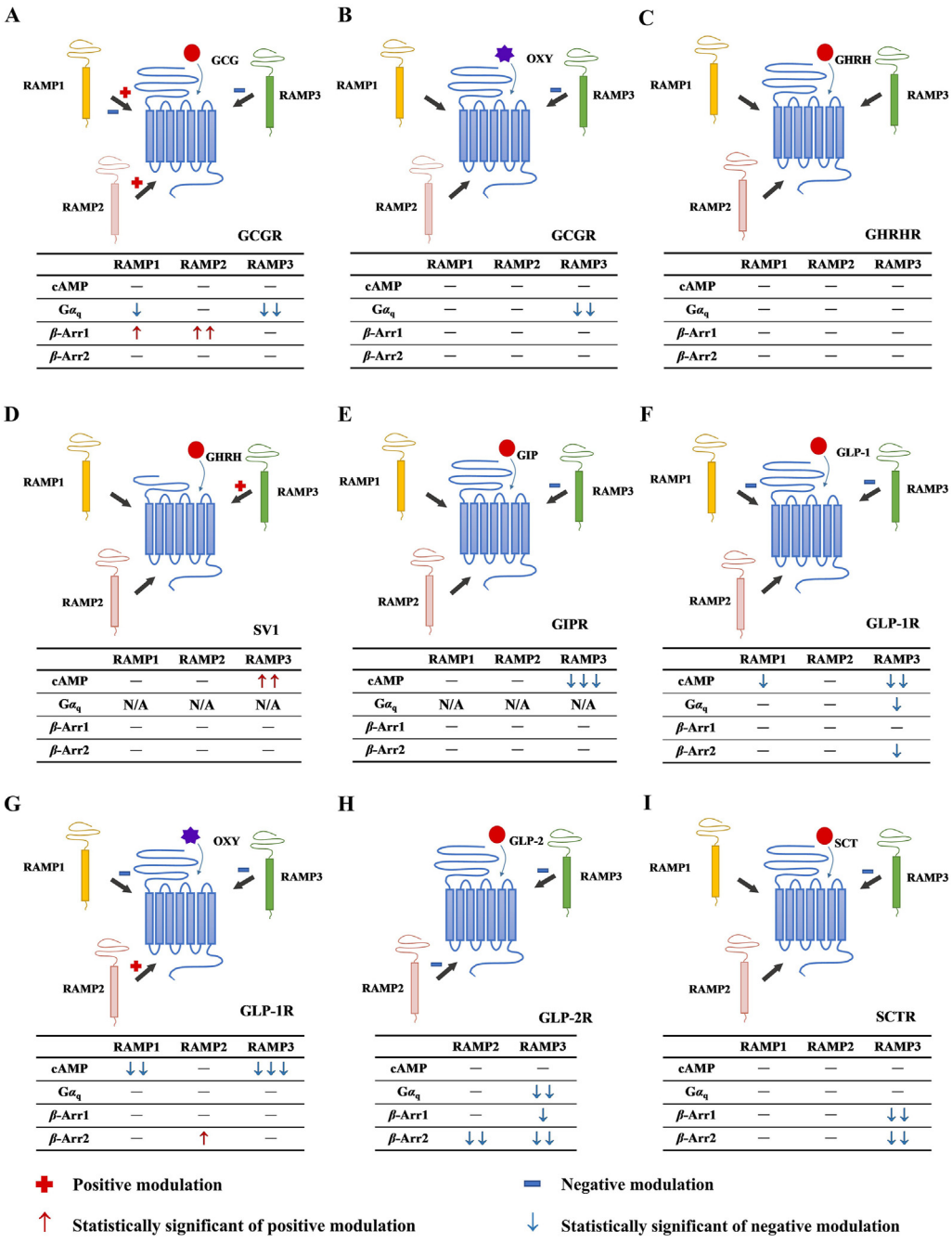


Figure 6 RAMP-mediated modulation of β-arrestin2 recruitment. β-Arrestin2 (β-Arr2) recruitment was assessed in COS-7 cells using a BRET assay. β-Arrestin2 level with each RAMP was normalized to the maximal response in the absence of RAMP at β_2-AR (C), GCGR (D and E), GHRHR (F), SV1 (G), GIPR (H), GLP-1R (I and J), GLP-2R (K) and SCTR (L). CLR was served as positive control and β_2-AR was the negative control. CLR was normalized to the measurement in the presence of RAMP1 (elicited by CGRP) and RAMP2 (elicited by adrenomedullin). Data shown are mean ± SEM from at least three independent experiments. Concentration−response curves were fitted to non-linear regression curves.

It's obvious that RAMPs broadly interact with the glucagon receptor family members thereby altering their functions. RAMP1 is in favor of G protein-related pathways, an observation consistent with that found in cells expressing VPAC2R[8], whereas RAMP2 mainly mediates β-arrestin recruitment. According to published studies, RAMP2 plays a major role in $G\alpha_{i/o/t/z}$ coupling to VPAC2R and $G\alpha_{i/q/12}$ coupling to corticotropin-releasing factor receptor 1[8], suggesting that its modulation is receptor specific. In addition, increased efficacy of RAMP2 on GCGR was seen in both HEK293T and CHO−K1 cells[38,39]. In GCGR-expressing CHO−K1 cells, RAMP2 attenuated $G\alpha_q$ signaling and abolished β-arrestin recruitment[38]. However, this result could not be reproduced in COS-7 cells. Unlike RAMPs 1 and 2, RAMP3 is indiscriminative of G protein or β-arrestin signaling and the modulation is of repressive nature. Furthermore, our internalization assay demonstrates that the function of RAMP3 is independent of receptor scavenging (Supporting Information Fig. S7).

The effect of RAMP also depends on ligand selectivity[40,41]. When activated by GLP-1, glucagon or oxyntomodulin (a dual-agonist), GLP-1R and GCGR mediated signaling pathways could be differentially modulated by RAMPs. Structural studies on different receptor−RAMP[30,31] and receptor−G protein[42] complexes suggest that there may exist two possible mechanisms of RAMP modulation: (i) directly affecting the ligand-receptor binding interface or (ii) indirectly changing receptor conformation[38].

RAMP may also participate in ligand-induced signal bias. Compared to GLP-1, oxyntomodulin exhibits a bias towards ERK1/2 phosphorylation over cAMP, with similar preference for cAMP relative to iCa^{2+} signaling. It also displays a stronger



A	RAMP1	RAMP2	RAMP3
cAMP	–	–	–
$G\alpha_q$	↓	–	↓↓
β-Arr1	↑	↑↑	–
β-Arr2	–	–	–

B	RAMP1	RAMP2	RAMP3
cAMP	–	–	–
$G\alpha_q$	–	–	↓↓
β-Arr1	–	–	–
β-Arr2	–	–	–

C	RAMP1	RAMP2	RAMP3
cAMP	–	–	–
$G\alpha_q$	–	–	–
β-Arr1	–	–	–
β-Arr2	–	–	–

D	RAMP1	RAMP2	RAMP3
cAMP	–	–	↑↑
$G\alpha_q$	N/A	N/A	N/A
β-Arr1	–	–	–
β-Arr2	–	–	–

E	RAMP1	RAMP2	RAMP3
cAMP	–	–	↓↓↓
$G\alpha_q$	N/A	N/A	N/A
β-Arr1	–	–	–
β-Arr2	–	–	–

F	RAMP1	RAMP2	RAMP3
cAMP	↓	–	↓↓
$G\alpha_q$	–	–	↓
β-Arr1	–	–	–
β-Arr2	–	–	↓

G	RAMP1	RAMP2	RAMP3
cAMP	↓↓	–	↓↓↓
$G\alpha_q$	–	–	–
β-Arr1	–	–	–
β-Arr2	–	↑	–

H	RAMP2	RAMP3
cAMP	–	–
$G\alpha_q$	–	↓↓
β-Arr1	–	↓
β-Arr2	↓↓	↓↓

I	RAMP1	RAMP2	RAMP3
cAMP	–	–	–
$G\alpha_q$	–	–	–
β-Arr1	–	–	↓↓
β-Arr2	–	–	↓↓

Figure 7 Characterization of RAMP interaction with the glucagon receptor family members. Interaction and modulation of RAMP at GCGR (A and B), GHRHR (C), SV1 (D), GIPR (E), GLP-1R (F and G), GLP-2R (H) and SCTR (I). Positive or negative modulations of each receptor−RAMP pair that achieved statistical significance are presented in tables below. GCG, glucagon; OXY, oxyntomodulin; SCT, secretin; β-Arr1, β-arrestin1; β-Arr2, β-arrestin2; N.A., no robust stimulation was detected at the highest concentration.

preference for β-arrestin1/2 recruitment relative to GLP-1[14,43,44]. In this study, both RAMP1 and RAMP3 showed low potency for cAMP production while RAMP2 enhanced β-arrestin2 recruitment at GLP-1R. This feature would promote the bias of oxyntomodulin towards β-arrestins or ERK1/2 phosphorylation over cAMP relative to GLP-1.

Both the glucagon receptor family members and RAMPs are key players of the metabolic and endocrine systems[10]. They have an overlap organ distribution including the lung, pancreas, kidney, brain, heart and liver[45−50]. Clearly, in-depth analysis of the relationship between GPCR−RAMP pair distribution and physiological relevance is required to understand the significance of RAMP modulation.

5. Conclusions

This present work describes the interactions of RAMPs with members of the glucagon receptor family of GPCRs. Through

verification of previously published RAMP interactome and identification of new GPCR−RAMP pairs, we were able to reveal that RAMP modulates both G protein dependent and independent signaling pathways in a receptor-specific manner.

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Author contributions

Lijun Shao, Yan Chen, Shikai Zhang and Zhihui Zhang performed the experiments; Lijun Shao, Yan Chen, Yongbing Cao, Dehua Yang and Ming-Wei Wang analyzed the data; Dehua Yang supervised functional studies; Lijun Shao, Yan Chen and Ming-Wei Wang drafted the manuscript; Ming-Wei Wang designed the experiments, oversaw the project and finalize the manuscript with inputs from all the authors.

Conflicts of interest

The authors declare no competing interests.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.07.028.

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