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Receptor Activity-Modifying Proteins Differentially Modulate the G Protein-Coupling Efficiency of Amylin Receptors

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Receptor activity-modifying proteins (RAMPs) 1, 2, and 3 are prototypic G protein-coupled receptor accessory proteins that can alter not only receptor trafficking but also receptor phenotype. Specific RAMP interaction with the calcitonin receptor (CTR) generates novel and distinct receptors for the peptide amylin; however, the role of RAMPs in receptor signaling is not understood. The current study demonstrates that RAMP interaction with the CTRa in COS-7 or HEK-293 cells leads to selective modulation of signaling pathways activated by the receptor complex. There was a 20- to 30-fold induction in amylin potency at CTR/RAMP1 (AMY_1) and CTR/RAMP3 (AMY_3) receptors, compared with CTR alone, for formation of the second-messenger cAMP that parallels an increase in amylin binding affinity. In contrast, only 2- to 5-fold induction of amylin potency was seen for mobilization of intracellular Ca^{++} or activation of ERK1/2. In addition, in COS-7 cells, the increase in amylin potency for Ca^{++} mobilization was 2-fold greater for AMY_3 receptors, compared with AMY_1 receptors and this paralleled the relative capacity of overexpression of $G_{\alpha q}$ proteins to augment induction of high affinity ^{125}I-amylin binding. These data demonstrate that RAMP-complexed receptors have a different signaling profile to CTRs expressed in the absence of RAMPs, and this is likely due to direct effects of the RAMP on G protein-coupling efficiency. (*Endocrinology* 149: 5423–5431, 2008)

G PROTEIN-COUPLED RECEPTORS (GPCRs) are the largest family of cell surface receptors; they play roles in virtually every physiological system and are implicated in most major diseases. Recently increasing attention has focused on the role of accessory proteins in the modulation of GPCR function (1–3). Receptor activity-modifying proteins (RAMPs) are prototypical accessory proteins that interact with specific receptors to alter their function including, in a receptor-dependent manner, cell surface expression, binding phenotype, internalization, and recycling (4, 5). The most characterized partners for RAMP interaction are the calcitonin (CT) and calcitonin receptor-like receptors that, with RAMPs, yield receptors for calcitonin family peptides, including CT, amylin, calcitonin gene-related peptide (CGRP), and adrenomedullin.

Amylin, a 37-amino acid peptide, is cosecreted with insulin from the pancreatic β-cells after food intake and has a range of effects on a number of different tissues to modulate nutritional status. It is a potent inhibitor of vagally mediated gastric emptying and has additional effects including reduced appetite, reduced postprandial glucagon secretion, and the inhibition of insulin-stimulated glycogen production in skeletal muscle (6, 7). The stable amylin analog, pramlintide, has recently received Food and Drug Administration approval for adjunct treatment of type 2 diabetes (7). Amylin is related to the other CT peptide family members and shares a number of actions with the other peptides, particularly CT (4). Nonetheless, CT has a distinct set of physiological actions, most notably to regulate blood calcium levels by inhibiting osteoclast mediated bone resorption and stimulating renal calcium clearance. It is commonly used for the therapeutic treatment of hypercalcemic conditions, including Paget's disease and osteoporosis (8).

Amylin receptors are generated from the CT receptor (CTR) gene product when coexpressed with RAMPs (9). RAMPs constitute a unique family of type I transmembrane proteins, comprising RAMP1, RAMP2, and RAMP3. Each RAMP possesses a large extracellular N-terminal domain, a single transmembrane α-helix and a small intracellular C-terminal domain. Although RAMPs share a common basic structure, including four conserved cysteines in the N terminus, they share only a relatively low (~30%) amino acid sequence identity. Originally discovered by McLatchie *et al.* (10) during attempts to clone the receptor for CGRP, RAMPs were shown to chaperone the calcitonin receptor-like receptor (CL-R) to the cell surface to form high-affinity CGRP and adrenomedullin receptor receptors. Unlike CL-R, CTR when expressed alone, traffics to the cell surface and functions as a high-affinity receptor for CT peptides. However, when coexpressed with RAMP1, RAMP2, or RAMP3, the CTR/RAMP complexes generate pharmacologically distinct amylin receptors, AMY_1, AMY_2, and AMY_3, respectively (4). Hence, RAMPs can act as a pharmacological

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Abbreviations: AMY, Amylin receptor; CGRP, calcitonin gene-related peptide; CL-R, calcitonin receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HA, hemagglutinin; HBSS, Hanks' buffered saline solution; hCT, human calcitonin; HEK-293, human embryonic kidney-293; PI, phosphoinositol; PKA, phosphokinase A; PKC, phosphokinase C; PLC, phospholipase C; RAMP, receptor activity modifying protein; rAmy, rat amylin.

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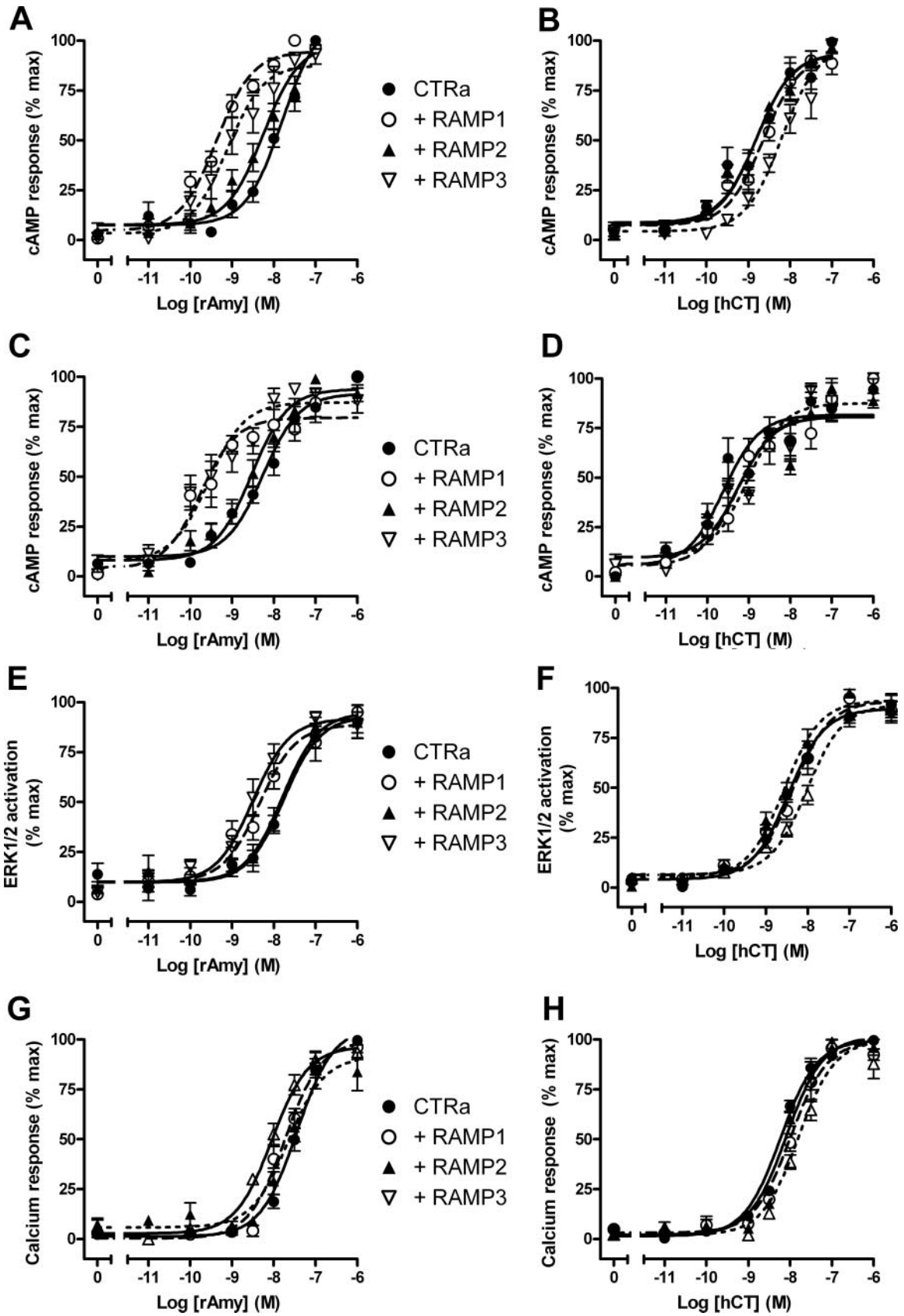

FIG. 1. Measurement of rAmy- or hCT-induced signaling activation via the CTa receptor in the absence or presence of RAMPs. COS7 cells were cotransfected with CTRa and either RAMP1 (*open circles*), RAMP2 (*filled triangles*), RAMP3 (*open inverted triangles*), or pcDNA control (*filled circle*)

switch for these two GPCRs, providing a sophisticated and novel mechanism for modulating receptor phenotype.

To date, there have been only limited studies looking at the potential role of RAMPs in modulation of CL-R or CTR signaling. Coexpression of RAMPs with the predominant receptor isoform CTRa had no effect on the magnitude of cAMP accumulation or phosphatidylinositol (PI) hydrolysis in COS-7 cells (11). Despite that early study, it is clear that the ability of RAMP2 to form functional amylin receptors is dependent on cellular background and on receptor isoform. For instance, CTRa expressed with RAMP2 generated an amylin receptor in CHO-P cells but only very weakly induced an amylin response in COS-7 cells (12). Furthermore, RAMP2 coexpressed with the CTRb isoform (differing to CTRa by a 16-aa insert in the first intracellular loop) was able to induce amylin receptors in COS7 cells (12), whereas the CTRa isoform effectively cannot. Interestingly, there is no significant difference in peptide binding between the two receptor isoforms, yet the presence of this loop does impair G protein coupling and signaling in a cell-specific manner (13, 14). Differences in the ability or availability of particular G proteins to interact with specific RAMP receptor heterodimers may account for the alterations in pharmacological phenotype and cell type specificity. This theory is supported by evidence that overexpression of $G_{\alpha s}$ proteins can modify the formation of functional RAMP/CTRa complexes in COS-7 cells (15).

The current study demonstrates that RAMP interaction with the CTRa leads to selective modulation of signaling pathways activated by the receptor complex. There was a marked induction in amylin potency at AMY_1 and AMY_3 receptors for formation of the second messenger cAMP that parallels the increase in amylin binding affinity. In contrast, only very weak induction of amylin potency was seen for mobilization of intracellular Ca^{2+} or activation of ERK 1/2. In addition, subtle differences in the effect of RAMP1 *vs.* RAMP3 were observed that were also seen in the relative capacity of overexpression of Gα proteins to augment induction of high-affinity amylin binding. These data demonstrate that RAMP-complexed receptors have a different signaling profile to CTRs expressed in the absence of RAMPs, and this is likely due to direct effects of the RAMP on G protein-coupling efficiencies.

Materials and Methods

Materials

Human calcitonin (hCT) and rat amylin (rAmy) were purchased from Auspep (Parkville, Victoria, Australia). Rat amylin is used in preference to human amylin due to the amyloidogenic property of human amylin. $CGRP_{8-37}$ was from Bachem (Bubendorf, Switzerland). AC187 was a gift from Dr. Andrew Young (Amylin Pharmaceuticals Inc., La Jolla, CA). BSA, isobutyl methylxanthine, and poly-L-lysine were from Sigma (St. Louis, MO). Amplified luminescent proximity homogenous assay-screen cAMP kits were purchased from PerkinElmer (Boston, MA), ERK Surefire assay kits were a kind gift from TGR Bioscience (Adelaide, Australia). Fluo-4 label was purchased from Molecular Probes (Invitrogen, Carlsbad, CA). DMEM and HEPES were from Invitrogen; fetal bovine serum (FBS) was from TRACE Biosciences (Sydney, Australia). Cell culture plasticware was from Nunc (Roskilde, Denmark), and Metafectene was from Biontex supplied by Scientifix (Melbourne, Australia). ^{125}I rat amylin was prepared using Bolton-Hunter reagent; 2000 Ci/mmol from Amersham (Buckinghamshire, UK), and purified by reverse-phase HPLC as described previously (16). ^{125}I-labeled goat antimouse IgG was obtained from PerkinElmer. The inhibitors H-89, U-73122, PD98059, U0126, wortmannin, and staurosporine were purchased from BIOMOL International (Plymouth Meeting, PA). Tyrphostin AG1478, Rö-31–8220, and ET-18-OCH_3 were purchased from Calbiochem (La Jolla, CA). Pertussis toxin and forskolin were purchased from Sigma-Aldrich (St. Louis, MO).

cDNA constructs

The preparation of cDNA with a double-hemagglutinin (HA) epitope tag at the N terminus of human CTa receptor (leucine^{447} variant) has been described previously (17). Human RAMP1, RAMP2, and RAMP3 cDNA constructs were a gift from Dr. Steven Foord (GlaxoSmithKline, Stevenage, UK) (10). cDNA for $G_{\alpha s}$ (short), $G_{\alpha i2}$, $G_{\alpha oA}$, and $G_{\alpha q}$ were from the UMR cDNA resource center (www.cdna.org). All constructs had been subcloned into the pcDNA3.1 mammalian expression vector (Invitrogen).

Cell culture and transfection

Green monkey kidney COS-7 cells were maintained in DMEM supplemented with 5% FBS and maintained at 37 C in a humidified atmosphere of 5% CO_2. Human embryonic kidney-293 (HEK-293) cells were maintained in DMEM supplemented with 10% FBS and maintained at 37 C in a humidified atmosphere of 5% CO_2. For competition binding assays, cells were seeded in 48 (1 cm^2)-well plates at a density of about 125,000 cells/well. The following day, when cells were 90–100% confluent, they were transfected using 0.75 μl/cm^2 of Metafectene with 50 ng and 75 ng/cm^2 of CTRa and RAMP cDNA, respectively, as previously described (18). For analysis of the effect of overexpression of G_α protein on RAMP-induced amylin binding, cells were seeded into 24-well plates to achieve a final density of about 250,000 cells/well. Cells were incubated under growth conditions for a further 36 h before being used in radioligand binding assay. For the functional assays, cells were seeded in 75-cm^2 flasks and grown overnight to about 90% confluence. HEK-293 cells were grown for 36 h to 90% confluence. Each flask of COS-7 cells was transfected with 3.2 μg of CTRa and 4.6 μg of RAMPs or pcDNA3-1 cDNA using 30 μl of metafectene per flask. Each flask of HEK-293 cells was transfected with 3.75 μg of CTRa and 5.7 μg of RAMP or pcDNA3-1 cDNA using 60 μl of metafectine per flask. Cells were incubated and recovered in growth media as described above. Cells were harvested 16 h after transfection and seeded for use in cAMP, ERK1/2 phosphorylation, and Ca^{2+} mobilization assays or for antibody binding experiments. The 96-well plates seeded with HEK-293 cells were poly-D-lysine treated before use. Allowing 16 h incubation for cells to adhere, cells were subsequently serum starved for a further 24 h before use in the functional assays.

Measurement of cAMP

Intracellular cAMP levels were determined using the AlphaScreen cAMP kit (PerkinElmer Life Sciences). On the day of assay, cells were harvested and assayed as previously described (18). Each assay point was performed in triplicate, and the quantity of cAMP generated was calculated from the raw data using a cAMP standard curve. For antagonist experiments, antagonist peptides were coadministered with agonist ligands as previously described (18).

Radioligand binding

Cells transfected in 48-well plates and incubated for approximately 36 h were assayed for ^{125}I-rat amylin binding. Cells were incubated in binding buffer [DMEM with 0.3% (wt/vol) BSA] containing approximately 120 pM ^{125}I-rat amylin in the absence (total binding) or presence

and cells were assayed 64 h afterward. The *left-hand panels* represent dose responses to rAmy stimulation and the *right-hand panels* to hCT stimulation. A and B, Agonist-mediated cAMP accumulation, measured after 30 min stimulation with rAmy or hCT, respectively. C and D, cAMP accumulation after 5 min stimulation. E and F, Graphed changes in ERK1/2 phosphorylation after agonist stimulation for 5 min; the time point of peak response determined from time-course assays (data not shown). Graphs (G and H) show the Fluo4-measured intracellular Ca^{2+} release. Data are presented as mean ± SEM, n = 4–9.

of increasing concentrations of unlabeled peptide (10^{-11} to 10^{-6} M). Cells were incubated for 1 h at 37 C before being washed with 250 μl PBS and then solubilized with 250 μl of 0.5 M NaOH. The cell lysate was collected and counted in a Packard γ-counter (75% efficiency) to determine bound radioactivity. Experiments were performed in duplicate (n = 4). To assess the effect of overexpression of G_α proteins on induction of AMY receptor phenotype, cells were plated into 24-well plates and transfected with CTRa (100 ng) and one of the four ($G_{\alpha s}$, $G_{\alpha i2}$, $G_{\alpha oA}$, $G_{\alpha q}$) G_α subtypes (150 ng) together with either pcDNA3.1 empty vector or one of RAMP1, RAMP2, or RAMP3 (100 ng). Whole cells were assayed for ^{125}I-rat amylin binding 48 h after transfection by incubating transfected cells with radioligand (80 pM) in the absence (total binding) or presence of 10^{-6} M unlabeled rAmy (nonspecific binding). Experiments were performed in triplicate (n = 3–5).

Measurement of cell surface expression of HA-CTR by antibody binding

Cell surface expression of HA-tagged CTa receptor was determined as previously described (18) about 48 h after transfection using mouse anti-HA (12CA5) antibody and ^{125}I-labeled goat antimouse IgG secondary.

Calcium mobilization assay

Transfected cells were seeded in poly-L-lysine-coated 96-well plates at a density of 50,000 cells/well, incubated overnight, and serum starved for a further 24 h. Cells were washed three times with a modified Hanks' buffered saline solution [HBSS; containing (in millimoles): NaCl 150; KCl 2.6; $MgCl_2$ 1.18; D-glucose 10; HEPES 10; $CaCl_2$ 2.2; probenecid 2, and 0.5% (wt/vol) BSA]. In light-diminished conditions, 100 μl of wash solution were added containing the cell-permeant Ca^{2+} fluorophore, Fluo-4/AM (10 μM), and incubated for 1 h at 37 C. The fluorophore solution was aspirated from the wells, and cells were washed twice and then incubated for 30 min in modified HBSS at 37 C. The assay plate was transferred to a FlexStation (Molecular Devices, Palo Alto, CA), which performed the robotic addition of ligands (10 × stocks in modified HBSS). Receptor-mediated changes in intracellular Ca^{2+} concentration were immediately recorded by the FlexStation using an excitation wavelength of 485 nm and emission wavelength of 520 nm. Data were collected for each well every 1.52 sec for a total of 135 sec.

ERK phosphorylation assay

Transfected cells were seeded in 96-well plates at a density of 50,000 cells/well and incubated for 16 h before being serum starved overnight. On the day of assay, cells were pretreated with buffer or inhibitors (at the concentrations specified) and then stimulated with agonist at 37 C. Concentrations of inhibitors used in this study were determined previously (19, 20), by inhibitor concentration response curves, or sourced from the literature. Inhibitors were dissolved in dimethylsulfoxide, and the final concentration of solvent in the assay was less than 1% and this did not affect the assay. Time-course results demonstrated a peak response at 5 min for all receptor complexes after agonist stimulation; this time point was subsequently used in concentration-response studies. ERK1/2 phosphorylation was measured using the AlphaScreen-based ERK SureFire assay kit as previously described (19). Data are expressed as fold change from basal (control treated cells). For antagonist experiments, agonist and antagonist peptides were coadministered to the wells.

Data analysis

Competition binding data and cAMP, ERK1/2 phosphorylation, and Ca^{2+} mobilization concentration-response data were analyzed via nonlinear regression using PRISM version 4.3 (GraphPad Software, San Diego, CA). In all instances, data shown are the mean ± SE. Comparisons between data were performed by one-way ANOVA; *post hoc* testing was via either Dunnett's test for comparisons with control or Tukey-Kramer's test for multiple comparisons. Statistical analysis was performed within the Prism 4.3 environment. Unless otherwise stated, values of $P < 0.05$ were taken as significant. Potency data are presented as negative log molar (p) as errors are log normally distributed.

Results and Discussion

The CTRa functionally couples to multiple effector pathways including the proximal second messengers, cAMP and Ca^{2+}, and the more distal effector, phosphorylated-ERK1/2 (8, 13, 14). We therefore examined the ability of RAMPs to induce amylin receptors that are linked to these pathways.

We first compared the relative ability of amylin, acting via RAMP/CTR complexes, to induce cAMP accumulation and stimulate ERK1/2 phosphorylation. CTRa was coexpressed in COS-7 cells with RAMP1, RAMP2, RAMP3, or vector control plasmid, and induction of cAMP accumulation or ERK1/2 phosphorylation was assessed in parallel. In agreement with previous findings in this cellular background, RAMP1 and RAMP3 induced a marked increase in amylin potency for cAMP production (27- and 21-fold, respectively), compared with CTRa receptor and vector, whereas RAMP2 had only a weak effect (Fig. 1A and Table 1) (9, 12). Responses to hCT were not affected by RAMP1 or RAMP2 but decreased (~3-fold) with RAMP3, presumably due to reduction in free CTRa availability (18) (Fig. 1B). In contrast to cAMP production, the potency of amylin in stimulating ERK1/2 phosphorylation in CTRa/RAMP coexpressing cells was much smaller (3.8- and 4.7-fold potency increase for RAMP1 and RAMP3, respectively) (Fig. 1E and Table 1).

TABLE 1. Agonist potency estimates in COS-7 cells

	cAMP (30 min)	Δ Change	cAMP (5 min)	Δ Change	ERK1/2 (5 min)	Δ Change	Ca^{2+}	Δ Change
rAmy								
CTRa + pcDNA3	7.80 ± 0.11 (5)		8.26 ± 0.11 (5)		7.76 ± 0.11 (7)		7.44 ± 0.06 (9)	
CTRa + RAMP1	9.23 ± 0.07 (6)[a]	27	9.76 ± 0.15 (5)[a]	31	8.34 ± 0.13 (8)[a]	3.8	7.73 ± 0.07 (9)[a]	2.0
CTRa + RAMP2	8.25 ± 0.10 (6)[a,b]	2.8	8.53 ± 0.09 (5)[b]	1.8	7.83 ± 0.10 (8)[b]	1.2	7.66 ± 0.14 (4)	0.7
CTRa + RAMP3	9.12 ± 0.13 (6)[a]	21	9.57 ± 0.14 (5)[a]	20	8.43 ± 0.11 (8)[a]	4.7	8.04 ± 0.08 (9)[a,c]	4.0
hCT								
CTRa + pcDNA3	8.80 ± 0.12 (5)		9.61 ± 0.16 (4)		8.47 ± 0.09 (8)		8.23 ± 0.04 (9)	
CTRa + RAMP1	8.64 ± 0.11 (6)		9.28 ± 0.16 (4)		8.35 ± 0.07 (8)		7.98 ± 0.09 (4)	
CTRa + RAMP2	8.82 ± 0.09 (6)		9.27 ± 0.18 (4)		8.56 ± 0.07 (8)		8.08 ± 0.08 (4)	
CTRa + RAMP3	8.20 ± 0.09 (6)[a]		9.03 ± 0.13 (4)		8.02 ± 0.08 (8)[a]		7.80 ± 0.08 (4)[a]	

Shown are the pEC_{50} values for peptide-induced cAMP production (for both 30 and 5 min agonist stimulation), ERK1/2 activation, and intracellular Ca^{2+} mobilization. Data are presented as mean ± SEM. The number of individual experiments analyzed is shown in *parentheses*. Fold Δ, Fold change in amylin potency when comparing potency for CTRa/RAMP1 complexes with CTRa + pcDNA3.

[a] Significantly different from CTRa control.

[b] Significantly different from CTRa/RAMP1 and CTRa/RAMP3.

[c] Significantly different from CTRa/RAMP1 and CTRa/RAMP2. One-way ANOVA with Tukey-Kramer multiple comparisons *post hoc* test ($P < 0.05$).

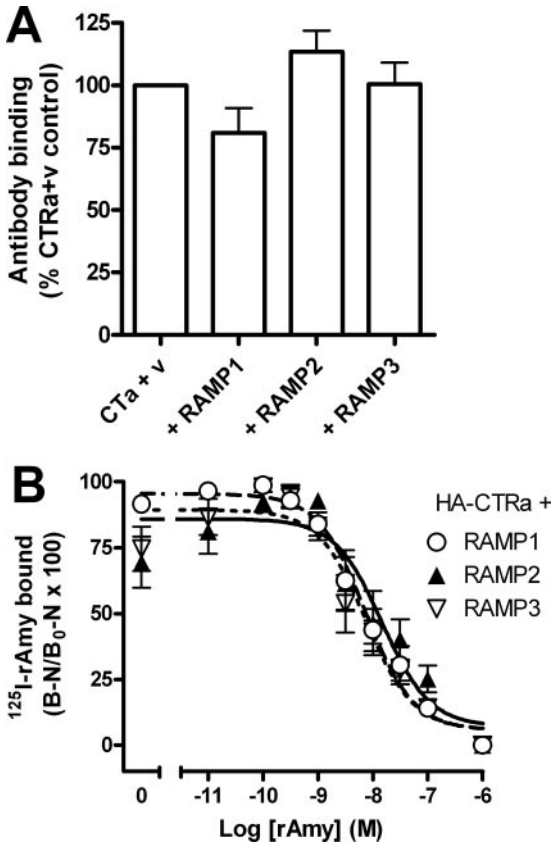

FIG. 2. Competition binding and cell surface receptor labeling for HA-CTa receptor in the absence or presence of RAMPs. A, Cell surface expression of CTRa protein. COS-7 cells are transiently transfected with CTRa and pcDNA3 empty vector (v) or CTRa and RAMP1, RAMP2, or RAMP3, measured by binding anti-HA antibody to the 2 × HA-N-terminal-tagged CTRa followed by detection with incubation of a ^{125}I-labeled goat antimouse IgG secondary antibody. Data are expressed as a percentage of the binding of ^{125}I-IgG antibody to cells expressing the CTRa protein alone. Data are mean ± SEM from eight independent experiments. B, Peptide competition binding for ^{125}I-rAmy binding to CTRa and RAMP1, RAMP2, or RAMP3. Data were mean ± SEM of four separate experiments with duplicate repeats. B, ^{125}I-rAmy. B_0, Total binding in the absence of cold peptide; N, nonspecific binding (measured in the presence of 10^{-6} M peptide).

Given the marked divergence in potency between the two signaling pathways we sought to establish whether the small increase in Amy potency for ERK activation was via the same receptor mediating the larger cAMP response. Therefore, we performed concentration-response analysis of amylin-induced ERK1/2 phosphorylation in the presence of $CGRP_{8-37}$, a selective antagonist that is able to differentiate between CTRa and AMY receptors due to substantial differences between the affinities of this antagonist for these receptor subtypes (18). $CGRP_{8-37}$ caused a rightward shift of the amylin dose-response curve at AMY_1 (CTRa + RAMP1) and AMY_3 (CTRa + RAMP3) receptors but not at CTRa (alone), whereas the nonselective antagonist AC187 right-shifted both hCT and amylin responses at each of the receptor complexes. The values of the negative logarithms of the antagonist equilibrium dissociation constant for the antagonist $CGRP_{8-37}$ at the AMY_1 and AMY_3 receptors were 6.8 ± 0.2 and 6.4 ± 0.4, respectively, and this is equivalent to those reported previously for cAMP accumulation (18). Thus, whereas the change is small, the increase in amylin potency observed in ERK1/2 activation is via the RAMP/CTR complexes. Direct stimulation of adenylate cyclase with forskolin was ineffective at activating ERK1/2 (data not shown), suggesting that ERK activation in this system could not be stimulated by cAMP alone and must result from coupling to alternative pathways. Taken together, the above results raise the possibility that the coupling efficiencies of AMY_1 and AMY_3 receptors for different G protein subtypes differs from that of the CTRa.

We subsequently investigated the ability of CTRa and AMY receptors to couple to $G_{\alpha q}$ by measuring the downstream release of intracellular Ca^{2+}. Analogous to cAMP responses, hCT-induced intracellular Ca^{2+} mobilization was mostly unaffected by coexpression of RAMPs, with only a small decrease in potency seen with coexpression of RAMP3 (Fig. 1H). CTRa coexpression with RAMP1 or RAMP3 led to a weak increase in amylin potency for Ca^{2+} mobilization of 2- and 4-fold, respectively (Fig. 1G). Together with the data from ERK1/2 activation (Fig. 1E and Table 1), this suggests that the relative coupling of AMY receptors is much stronger to $G_{\alpha s}$ than to the alternative pathways investigated.

This large difference in coupling efficiency across the three effector pathways measured was not observed at the CTRa in the absence of RAMPs; when CTRa was expressed alone, the agonist potencies in the cAMP, ERK1/2, and Ca^{2+} mobilization were similar for both rAmy and for hCT (Table 1). When comparing the potency of amylin across the CTRa/RAMP com-

TABLE 2. Agonist potency estimates in HEK-293 transfected cells

	cAMP (30 min)	Δ Change	ERK1/2 (5 min)	Δ Change	Ca^{2+}	Δ Change
rAmy						
CTRa + pcDNA3	8.28 ± 0.23 (4)		7.53 ± 0.16 (4)		7.51 ± 0.14 (4)[a]	
CTRa + RAMP1	9.69 ± 0.28 (4)[a]	25.4	7.78 ± 0.10 (4)	1.8	8.22 ± 0.09 (4)[a]	4.0
CTRa + RAMP2	9.08 ± 0.31 (4)	6.2	7.57 ± 0.13 (4)	1.1	7.44 ± 0.11 (4)[b]	0.7
CTRa + RAMP3	9.92 ± 0.13 (4)[a]	42.9	8.09 ± 0.12 (4)[a]	3.6	8.23 ± 0.02 (4)[a]	4.1
hCT						
CTRa + pcDNA3	9.79 ± 0.13 (4)		7.89 ± 0.03 (4)		8.07 ± 0.27 (4)	
CTRa + RAMP1	9.88 ± 0.12 (4)		8.12 ± 0.18 (4)		7.65 ± 0.09 (4)	
CTRa + RAMP2	9.70 ± 0.25 (4)		8.09 ± 0.10 (4)		8.11 ± 0.05 (4)	
CTRa + RAMP3	8.78 ± 0.06 (4)[a]		7.83 ± 0.27 (4)		7.73 ± 0.17 (4)	

Shown below are the pEC_{50} values for peptide-induced cAMP production, ERK1/2 activation, and intracellular Ca^{2+} mobilization. Data are presented as mean ± SEM. The number of individual experiments analyzed is shown in *parentheses*. Fold Δ, Fold change in amylin potency when comparing potency for CTRa/RAMP1 complexes with CTRa + pcDNA3.

[a] Significantly different from CTRa control.

[b] Significantly different from CTRa/RAMP1 and CTRa/RAMP3. One-way ANOVA with Tukey-Kramer multiple comparisons *post hoc* test ($P < 0.05$).

plexes, we also found that amylin was more potent on AMY_3 than AMY_1 receptors for Ca^{2+} mobilization (Table 1), which is the reverse of that observed in cAMP accumulation in the COS-7 cell background.

To eliminate the possibility that the changes in amylin potency observed were due to differences in receptor expression, we measured cell surface receptor expression in parallel to the functional assays by immunodetection using antibodies against the HA tag on the N terminus of the CTRa. The presence of RAMPs did not significantly alter the amount of CTRa expressed on the cell surface (Fig. 2A). As a further control, we used unlabeled rAmy to competitively inhibit ^{125}I rAmy binding to cells coexpressing CTRa and RAMPs. The data illustrate that rAmy has approximately equal affinity for AMY_1, AMY_2, and AMY_3 receptors (Fig 2B) with pIC_{50} values of 8.13 ± 0.10, 7.83 ± 0.17, and 8.13 ± 0.12, respectively, although the level of induced binding was less with RAMP2; this is in agreement with previously published data in this cellular background (12, 21).

The cAMP, ERK1/2, and Ca^{2+} functional responses assessed in this study were measured over different time scales. The cAMP assay is an accumulation assay in which receptors are exposed to the agonist for 30 min before quantifying the amount of cAMP produced. In comparison, ERK1/2 phosphorylation is measured 5 min after agonist stimulation, whereas the Ca^{2+} mobilization is measured as peak response that occurs seconds after ligand exposure. For both ERK1/2

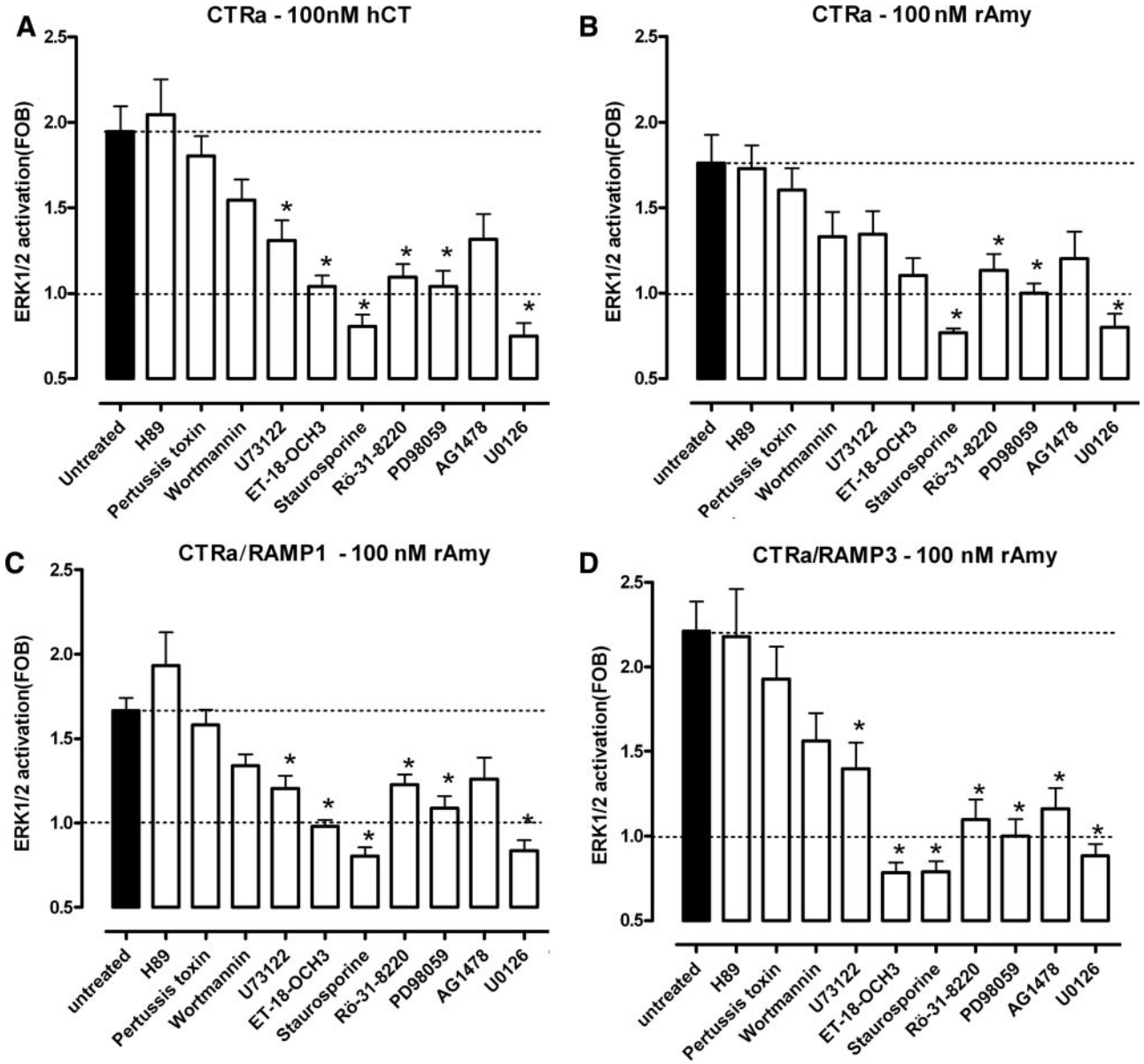

FIG. 3. Pharmacological inhibitor-based analysis of signaling components involved in activation of ERK1/2. Shown is peak (5 min) ERK1/2 phosphorylation in COS7 cells expressing CTRa and pcDNA3-1 (A and B), RAMP1 (C), or RAMP3 (D) after stimulation with 100 nM rAmy (A, C, and D) or hCT (B). Before stimulation with agonists, cells were preincubated with either buffer (untreated) or one of the following inhibitors; H-89 (10 μM, 1 h), pertussis toxin (100 ng/ml, 18 h), wortmannin (100 nM, 30 min), U73122 (10 μM, 30 min), ET-18-OCH_3 (100 μM, 30 min), staurosporine (1 μM, 30 min), Rö-31–8220 (10 μM, 30 min), PD98059 (20 μM, 30 min), Tyrphostin AG1478 (100 nM, 30 min), or U0126 (10 μM, 30min). Data are mean ± SEM, n = 3–4. *, Significantly different from vector control group; one-way ANOVA with Dunnett's *post hoc* test.

and Ca^{2+} assays, the ligand has not had sufficient time to equilibrate in the system before the measurement of downstream signaling and this may cause difficulties in comparing results of these assays with the cAMP assay, in which equilibrium can be reached. To ensure that the differences we observed were caused by RAMPs and not by a nonequilibrium effect, we repeated the cAMP accumulation assay, measuring cAMP production after only 5 min agonist treatment, equivalent to the agonist stimulation time of the ERK1/2 activation assay. The results demonstrate that the strong induction of amylin phenotype at the CTRa in the presence of RAMP1 and RAMP3 is maintained, with a 31- and 20-fold enhancement of potency, respectively, compared with CTRa alone (Fig. 1C). Interestingly, the pEC_{50} values for the 5-min cAMP assay are all consistently 2- to 6-fold greater than the 30-min assay, which is probably due to the nonequilibrated status of the system (Table 1). The data confirm that the greater induction of amylin phenotype seen in the cAMP assays, compared with the ERK1/2 phosphorylation and Ca^{2+} mobilization assays is not due to the assay conditions.

We subsequently extended these studies to examine signaling via CT and AMY receptors in an alternate cell background, HEK-293 cells. As seen for COS-7 cells, a strong induction of amylin-mediated cAMP signaling was observed with AMY_1 and AMY_3 receptors (25- and 43-fold, respectively; Table 2), but only weak induction of signaling was seen in ERK1/2 activation and Ca^{2+} mobilization assays (1.8- to 4.1-fold; Table 2). Interestingly, although AMY receptor-mediated ERK and Ca^{2+} signaling was relatively weak, there was greater fold induction seen in the Ca^{2+} *vs.* the ERK assay; this is the reverse of the pattern observed in COS-7 cells (Table 2 *vs.* Table 1). This suggests that some cell background-dependent modulation of signaling occurs. These data also suggest that the results demonstrated in COS-7 cells may be more generally relevant. Additional support for the differential coupling of CT and AMY receptors can be found in early studies on endogenously expressed amylin receptors in α-TSH thyrotroph cells. The α-TSH cell line has an AMY_{1a}-like receptor, having similar affinity for CGRP and amylin, in addition to an endogenous CTR. In this cell line, both cAMP and intracellular calcium mobilization were measured; however, amylin evoked responses only via the cAMP pathway (22), consistent with relatively weak coupling to calcium mobilization.

We next performed ERK1/2 phosphorylation and intracellular Ca^{2+} mobilization assays in the presence of selective signaling inhibitors to interrogate the pathway of activation for differing RAMP/receptor complexes. The use of inhibitors of protein kinase A (PKA) (H89; 10 μM, 1 h) and $G_{\alpha i/o}$ proteins (pertussis toxin; 100 ng/ml, 18 h) established that stimulation of ERK1/2 phosphorylation was independent of activation of PKA or $G_{\alpha i/o}$ proteins, the former being consistent with lack of forskolin-mediated activation of ERK1/2 in the COS-7 cells. Inhibition of both PI 3-kinase (wortmannin; 100 nM, 30 min) and PI-phospholipase C (PLC) (U73122; 10 μM, 30 min) led to partial inhibition of response, as did inhibition of the epidermal growth factor receptor tyrosine

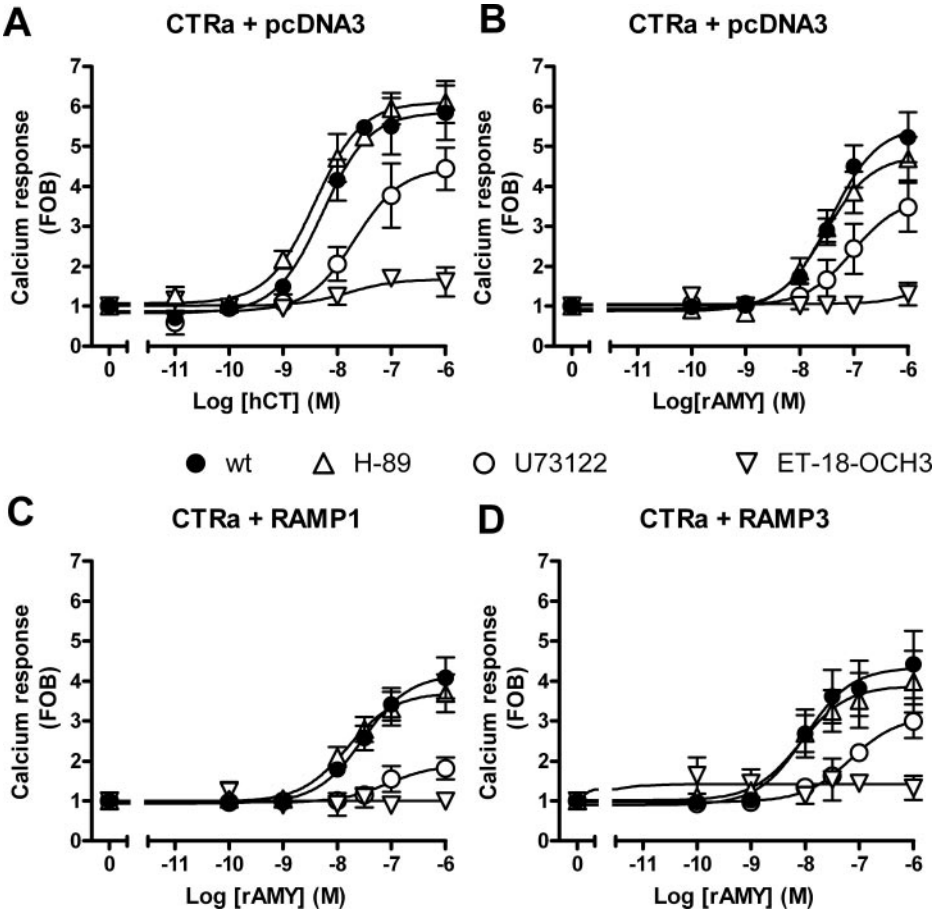

FIG. 4. rAmy-induced Ca^{2+} mobilization is differentially modulated by PLC inhibition in CTRa/RAMP1 *vs.* CTRa/RAMP3 expressing COS-7 cells. Ca^{2+} mobilization in response to increasing concentrations of hCT (A) or rAmy (B–D) in COS-7 cells transiently transfected with CTRa together with pcDNA3-1 (A and B), RAMP1 (C), or RAMP3 (D). Cells were pretreated with buffer (*filled circles*) or inhibitors for the times indicated before agonist stimulation in the continued presence of inhibitor. The inhibitors used were: H-89 (*open triangles*, PKA inhibitor; 10 μM, 1 h); U73122 (*open circles*, PLC inhibitor; 10 μM, 30 min); ET-18-OCH_3 (*open inverted triangles*, PLC, PI 3-kinase, PKC inhibitor; 100 μM, 30 min). Data are mean ± SEM, n = 3. FOB, Fold over basal. Maximum response (E_{max}) values (FOB) for the AMY_1 receptor in the absence and presence of U73122 were 4.17 ± 0.27, n = 5, and 1.91 ± 0.19, n = 3, respectively. E_{max} values for the AMY_3 receptor in the absence and presence of U73122 were 4.34 ± 0.40, n = 5, and 3.12 ± 0.20, n = 3, respectively. The effect of U73122 was significantly greater for the AMY_1 receptor, compared with the AMY_3 receptor or CTRa alone, $P < 0.05$; one-way ANOVA followed by Bonferri's multiple comparison test.

kinase (AG1478; 100 nM, 30 min), indicating that each of these pathways could contribute to the ERK response. Inhibition of protein kinase C (PKC; staurosporine; 1 μM, 30 min; Rö-318220; 10 μM, 30 min) led to the abolition of signaling, as did inhibition of MAPK kinase (U0126; 10 μM, 30 min; PD98059; 20 μM, 30 min), indicating that there is convergence on PKC and eventually MAPK kinase for activation. ET-18-OCH_3, which can inhibit PI-PLC, PI 3-kinase, and Raf (100 μM, 30 min) also abolished signaling. However, there was no significant difference in the relative contribution of the different signaling pathways to ERK activation between the CTRa alone or the AMY_1 or AMY_3 receptor complexes (Fig. 3). The inability to detect pathway differences in the activation of ERK is likely to be due, at least in part, to the strong background phenotype of the CTRa alone in the RAMP cotransfected cells coupled with only a weak induction of amylin phenotype for signaling to this pathway.

For Ca^{2+} mobilization, the PKA inhibitor H89 had no effect on responses to either hCT or amylin, whereas the broad-spectrum inhibitor ET-18-OCH_3 completely abolished signaling at each of the receptors (Fig. 4). In contrast, there were receptor-specific differences in the capacity of the PI-PLC inhibitor U73122 to inhibit responses. There was strong inhibition of signaling via the AMY_1 receptor complex (~72%), compared with weak inhibition for the CTRa alone or the AMY_3 receptor complex (30–40% inhibition).

The above signaling data implied that the different RAMP/CTR complexes were causing differential modulation of the ability of the receptor to interact with specific G proteins. To test this more empirically, we examined the

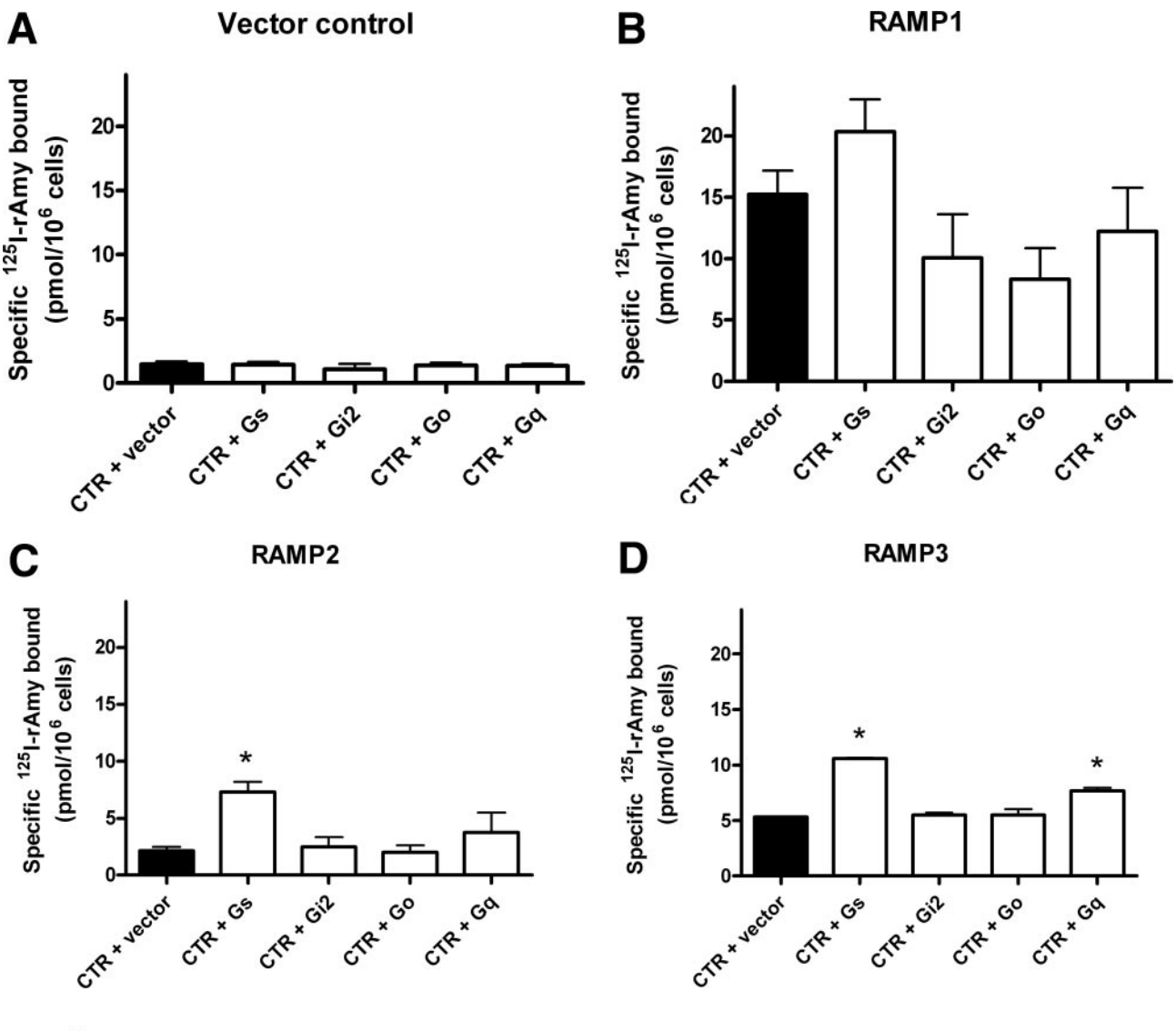

FIG. 5. Overexpression of Gα subunits differentially modulates induction of amylin receptor phenotypes by RAMPs. ^{125}I-rAmy binding to COS-7 cells cotransfected with CTRa (100 ng) and one of the four ($G_{\alpha s}$, $G_{\alpha i2}$, $G_{\alpha oA}$, $G_{\alpha q}$) G_α subtypes (150 ng) together with either pcDNA3.1 empty vector (A), RAMP1 (B), RAMP2 (C), or RAMP3 (D) DNA (150 ng). Whole cells were assayed for ^{125}I-rAmy binding 48 h after transfection by incubating transfected cells with radioligand (80 pM/well) in the absence (total binding) or presence of 10^{-6} M unlabeled rAmy (nonspecific binding). Specific binding was determined by subtracting nonspecific from total binding. Data are mean ± SEM, n = 3–5. *, Significantly different from vector control group; one-way ANOVA with Dunnett's *post hoc* test.

effect of over expression of different Gα protein subunits (G_s, G_{i2}, G_{oA}, G_q) on the ability of individual RAMPs to induce high affinity ^{125}I-rAmy binding. As previously reported in this cell background, RAMP1 and RAMP3 potently induced ^{125}I-rAmy binding, with the greatest effect seen with RAMP1, whereas RAMP2 generated only a low level of induced binding (Fig. 5). None of the G_α subunits modulated the binding to the CTRa expressed alone (Fig. 5A). Similarly, whereas there were trends for G_s to increase and G_o to decrease RAMP1-induced binding, neither of these was significant (Fig. 5B). In contrast, there was a large increase in RAMP2-induced binding in the presence of G_s but not other G_α proteins (Fig. 5C), whereas both G_s and G_q led to increased ^{125}I-rAmy binding with RAMP3 (Fig. 5D). Thus, these data support the proposition that individual RAMPs may each lead to a different profile of signaling from the CTR expressed alone.

Structurally, the three RAMPs have only a very short intracellular C-terminal tail of about 10 amino acids (10). Evidence for a direct role of this domain in the signaling specificity of RAMP receptor complexes arises from chimeras of RAMP1 and RAMP2 in which the potency of CGRP, a high-affinity ligand of RAMP1-complexed CTR but not RAMP2-complexed CTR, to stimulate cAMP production was contextual on the C-terminal domain present; CGRP had increased potency when the RAMP1 C terminus was present, despite an overt binding phenotype that was primarily influenced by the N-terminal domain present (23). Further evidence for involvement of the C terminus in G protein interaction arose from studies on C terminally truncated RAMPs; deletion of the last eight amino acids led to a marked loss in the capacity of RAMPs to induce high-affinity amylin receptors from CTRa receptors. This loss could be, at least partially, recovered by the overexpression of $G_{\alpha s}$ protein, indicating that the RAMPs were contributing directly to the ability of the receptor complexes to interact with G proteins (15). Intriguingly and consistent with the current study, truncation of the C terminus differentially affected individual RAMPs, and there were also differences in the sensitivity of the truncated RAMP/CTR complexes to recovery by $G_{\alpha s}$ overexpression.

In conclusion, this study demonstrates that RAMPs can differentially modulate the coupling efficiency of CTRa to various G proteins. This expands the repertoire of actions that RAMPs have in modulating GPCR function, and likely extends beyond the CTR investigated in the current work. This form of fine manipulation of receptor signaling provides new opportunities for development of novel therapeutic agents targeting RAMP complexed receptors.

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