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Distinct Receptor Activity-Modifying Protein Domains Differentially Modulate Interaction with Calcitonin Receptors

Madhara Udawela, George Christopoulos, Nanda Tilakaratne, Arthur Christopoulos, Anthony Albiston, and Patrick M. Sexton

Department of Pharmacology, Monash University, Victoria, Australia (P.M.S., A.C., G.C., N.T.); and Howard Florey Institute (P.M.S., M.U., A.A.) and Department of Pharmacology (A.C., M.U.), the University of Melbourne, Victoria, Australia

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ABSTRACT

Calcitonin receptors (CTRs) dimerize with receptor activity-modifying proteins (RAMPs) to generate high-affinity amylin (AMY) receptors; however, the relative contribution of individual RAMP domains to the formation of AMY receptors is poorly understood. We have used chimeras between RAMP1 and RAMP2 that specifically exchanged the N-terminal, transmembrane, or C-terminal domain and examined these in assays of [^{125}I]amylin binding or peptide-induced cAMP signaling in COS-7 cells transiently transfected with wild-type or chimeric RAMPs and human CTRa. The specificity of peptides in competition for [^{125}I]amylin binding was principally dictated by the N-terminal domain present in the chimeras; however, the maximal level of binding induced was dictated by the transmembrane domain present. This extended previous data (Zumpe et al., 2000) to provide a distinction between the transmembrane domain and the C terminus in this function. In contrast to the effects on binding, each of the RAMP domains played a role in the signaling phenotype of the receptors. In particular, the potency of calcitonin gene-related peptide (CGRP) was most influenced by the C-terminal domain present, in which the presence of the RAMP1 C-terminal domain led to increased potency over CTRa alone, whereas chimeras with the RAMP2 C-terminal domain did not induce increased CGRP potency. The data provide additional support for the importance of the N terminus in determining binding affinity but reveal a prominent role of the transmembrane domain in the strength of amylin binding and a unique role for the C terminus in signaling by peptides to stimulate cAMP production.

The definition of the G protein-coupled receptor (GPCR) phenotype has become increasingly complex with the demonstration of various protein-protein interactions leading to altered receptor pharmacology. A prime example of this is the modulation of GPCRs by receptor activity-modifying proteins (RAMPs) (Poyner et al., 2002; Udawela et al., 2004; Hay et al., 2006). RAMPs are a family of three type I transmembrane proteins that interact with a range of family B GPCRs, most notably the calcitonin receptor (CTR) and calcitonin receptor-like receptor (CLR), to affect various aspects of their behavior, which may include their cellular localization, signaling specificity, regulation, and profile of ligand interaction (Hay et al., 2006). For the CTR and CLR, RAMP interaction determines receptor specificity, with each individual RAMP engendering a different receptor phenotype upon interaction with either GPCR. These GPCR/RAMP heterodimeric complexes are recognized as the molecular units comprising the distinct amylin (AMY), adrenomedullin, calcitonin gene-related peptide (CGRP), and calcitonin (CT) receptor phenotypes (Poyner et al., 2002).

Recent work has investigated the molecular and structural basis for RAMP function and demonstrated that the large RAMP N-terminal domain is critical for interaction with CLR and for the resultant phenotype of RAMP/CLR complexes (Fraser et al., 1999; Kuwasako et al., 2001, 2003; Fitzsimmons et al., 2003). However, work with the CTR has revealed additional effects on phenotype that are dependent on cellular background in which coexpression of RAMP2 and CTRa (the predominantly human receptor splice variant) in Chinese hamster ovary-P but not COS-7 cells led to the induction of an AMY receptor phenotype (Tilakaratne et al., 2000). Phenotype differences were also seen between alternate splice variants of the CTR with a high level of AMY binding seen for RAMP2 complexes with the CTRb isoform, which

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AMY, amylin receptor phenotype; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; HA, hemagglutinin epitope tag; RAMP, receptor activity-modifying protein; sCT, salmon calcitonin; TMD, transmembrane domain; h, human; r, rat; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium.

has an additional 16 amino acids in intracellular loop 1 (Moore et al., 1995) in both Chinese hamster ovary-P and COS-7 cells (Tilakaratne et al., 2000). These experiments indicated that RAMP/GPCR complexes functionally interacted with other cellular proteins and therefore that the RAMP C terminus may be an important domain for RAMP function.

Studies on the binding of amylin to complexes of CTRa and N-terminal domain exchange chimeras of RAMP1 and RAMP2 suggested that whereas the N terminus was the key domain for binding specificity of peptides, the transmembrane domain and/or C terminus was important for the level of induced amylin binding and possibly for the stability of RAMP-receptor dimers (Zumpe et al., 2000).

This study further explores the distinct roles of the N-terminal, transmembrane, and C-terminal RAMP domains in AMY receptor function using chimeras between RAMP1 and RAMP2 that individually exchange each of these domains. The data provide additional support for the importance of the N terminus in determining binding affinity but reveals a prominent role of the transmembrane domain (TMD) in the strength of amylin binding and a unique role for the C terminus in signaling by peptides to stimulate cAMP production.

Materials and Methods

Human calcitonin (hCT), salmon calcitonin (sCT), human αCGRP, and rat amylin (rAMY) were purchased from Auspep (Parkville, VIC, Australia). Tissue culture reagents were from Invitrogen (Carlsbad, CA). Oligonucleotide primers were synthesized by GeneWorks (Adelaide, SA, Australia). *N*-succinimidyl-3-(4-hydroxy-[^{125}I]iodophenyl) propionate (Bolton-Hunter reagent; 200 Ci/mmol) was supplied by GE Healthcare (Little Chalfont, Buckinghamshire, UK). [^{125}I]rAMY (specific activity, 2000 Ci/mmol) was iodinated by the Bolton-Hunter method and purified by reverse-phase high-performance liquid chromatography as described previously (Bhogal et al., 1992).

cDNA Constructs. Expression clones of hCLR, HA-CLR, wild-type hRAMPs, and chimeric RAMP1/2 and RAMP2/1 (all in pcDNA3) were provided by Dr. S. M. Foord (Fraser et al., 1999). Double HA epitope-tagged human CTRa (HA-CTRa) was prepared as described previously (Pham et al., 2004). Transmembrane and C-terminal domain swap chimeras were created using the megaprimer polymerase chain reaction technique (Sarkar and Sommer, 1990; Barik and Galinski, 1991). The N-terminal/transmembrane domain junction for the chimeras was the common amino acid sequence aspartic acid-proline-proline at positions 113 to 115 of RAMP1 and 140 to 142 of RAMP2, and the transmembrane/C-terminal domain junction was the common amino acid sequence leucine-valine-valine at positions 136 to 138 of RAMP1 and 163 to 165 of RAMP2 (internal primers, 5′-ctgctggtgacggcacttgtagtatggagg-3′ and 5′-cccttcctcatcactctggtg-gtctggcag-3′). The outer primers were designed to incorporate a HindIII site upstream of and an XbaI site downstream of the RAMP sequence (5′-catcaagcttgccaccatggctcgggccctg-3′ and 5′-gcaatctagatatctacacaatgccctcagtg-3′ for RAMP1 and 5′-catcaagcttgccaccatggcctcgctc-3′ and 5′-gcaatctagatatctaggcctgggcctcactgtc-3′ for RAMP2). Polymerase chain reaction reactions were performed at an annealing temperature of 55°C for 30 cycles. Final full-length products were cloned directionally into the HindIII and XbaI restriction sites of pcDNA3.1 (Invitrogen). All constructs were confirmed by sequencing. The chimeras used in the study are represented schematically in Fig. 1.

Cell Culture and Transfections. COS-7 cells were routinely maintained in 175-cm^2 flasks at 37°C in a humidified atmosphere with 5% CO_2/95% air in complete DMEM supplemented with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 50 μg/ml fungizone. Transfections were carried out in serum and antibiotic-free DMEM using Lipofectamine (Invitrogen) or Metafectene (Scientifix, Cheltenham, VIC, Australia) when cells were ~70% confluent. Twenty-four-well plates were transfected with 100 ng of receptor and 150 ng of RAMP with 1 μl of lipid; 75-cm^2 flasks with 4 μg of receptor and 6 μg of RAMP with 20 μl of lipid; and 25-cm^2 flasks with 1 μg of receptor and 1.5 μg of RAMP with 8 μl of lipid.

Receptor Binding. To determine specific binding, 48 h after transfection in 24-well plates, COS-7 cells were incubated with ~100 pM [^{125}I]rAMY in binding buffer (DMEM containing 0.1% BSA) in the absence (total binding) or presence (nonspecific binding) of 1×10^{-6} M rAMY. After incubating at 37°C for 1 h, cells were washed with 1× phosphate-buffered saline and solubilized with 0.5 M NaOH. The radioactivity of the cell lysate was detected with a γ-irradiation counter. For competition binding, COS-7 cells were transfected in 75-cm^2 flasks and grown for 48 h, then harvested and resuspended in binding buffer (DMEM containing 0.1% BSA). Cells were added to 96-well plates (100,000 cells/well) with ~70 pM [^{125}I]rAMY and competing unlabeled peptides. After incubating for 1 h at 37°C, cells were harvested onto GF/C plates (coated with 0.5% polyvinylpyrolidone and 0.1% Tween 20) using a Tomtec harvester (Tomtec, Orange, CT). Plates were dried overnight and, after the addition of Microscint 0$^-$, counted on a TopCount counter (both from PerkinElmer Life and Analytical Sciences, Boston, MA). Experiments were performed with triplicate repeats.

cAMP Assays. Intracellular cAMP levels were determined using the AlphaScreen cAMP kit (PerkinElmer). Cells transfected in 25-cm^2 flasks were grown for 48 h and then serum-starved overnight. Cells were then harvested, counted, and incubated in stimulation

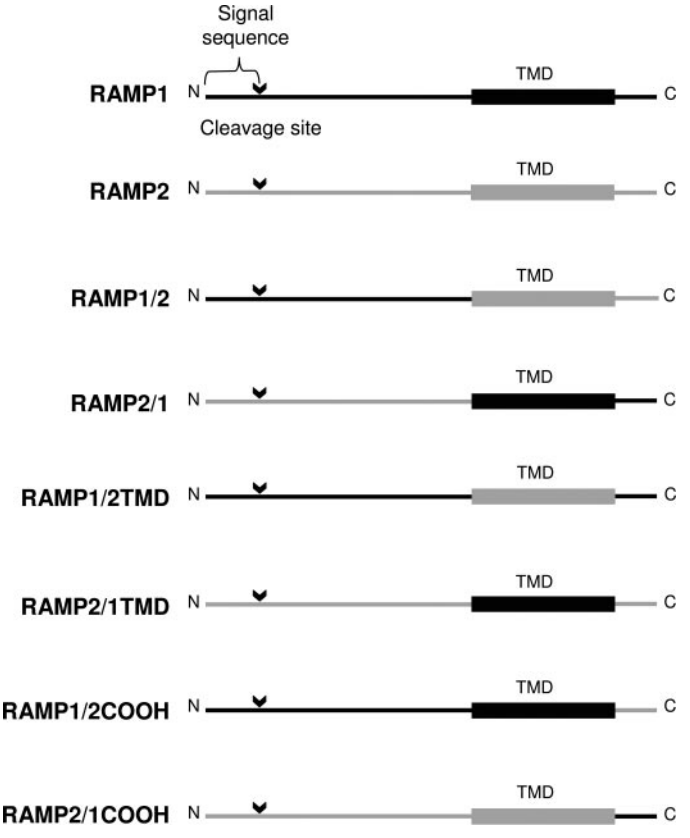

Fig. 1. Schematic representation of RAMP domain swap constructs showing the extracellular N-terminal domain containing signal sequence and cleavage site, the TMD, and the intracellular C-terminal domain. Regions depicted in black indicate RAMP1 sequence and in gray indicate RAMP2 sequence.

buffer (phenol red free media, 0.1% BSA, and 1 mM 3-isobutyl-1-methylxanthine) for 20 to 30 min. Cells were added to wells containing agonists at 5000 cells/well in 384-well plates. After stimulation for 30 min at 37°C, lysis buffer (5 mM HEPES, 0.3% Tween 20, and 0.1% BSA) was added to all wells, with subsequent steps performed as described previously (Pham et al., 2005). Each assay point was done in triplicate.

Data Analysis. A minimum of four independent repeats were performed for each of the above experiments, and the results are presented as mean ± S.E.M. Curve-fitting was done using Prism 4 (GraphPad Software Inc., San Diego, CA). Comparison of pIC_{50} or pEC_{50} values across all of the constructs was performed using the F test within Prism, where a value of $P < 0.05$ was considered significant across the data set. Post hoc comparison of chimeras with either the RAMP1-induced phenotype (for pIC_{50} values) or the CTa alone phenotype (for pEC_{50} values) was performed using an unpaired t test, where a value of $P < 0.05$ was considered significant.

Results

The Role of RAMP Transmembrane and Carboxy-Terminal Domains on the Induction of AMY Receptor Phenotype with CTRs. To determine the relative contribution of the RAMP transmembrane or C-terminal domains toward the induction of AMY binding, chimeras that individually exchange these two domains between RAMP1 and RAMP2 were tested for the induction of [^{125}I]rAMY binding (Fig. 2). The RAMP1/2 chimera, containing the N terminus of RAMP1 spliced to the TMD and C terminus of RAMP2, induced a low level of [^{125}I]rAMY binding, equivalent to that induced by RAMP2, whereas the reverse chimera RAMP2/1 induced a high level of AMY binding, equivalent to that induced by RAMP1 (Fig. 2). Chimeras containing the TMD of RAMP1 (RAMP2/1TMD; RAMP1/2COOH) induced high levels of [^{125}I]AMY binding, equivalent to RAMP1, whereas chimeras with the TMD of RAMP2 (RAMP1/2TMD; RAMP2/1COOH) gave equivalent [^{125}I]rAMY binding levels to RAMP2 (Fig. 2). Therefore, [^{125}I]AMY binding levels, at least in the context of these chimeras, were influenced principally by the TMD of RAMPs.

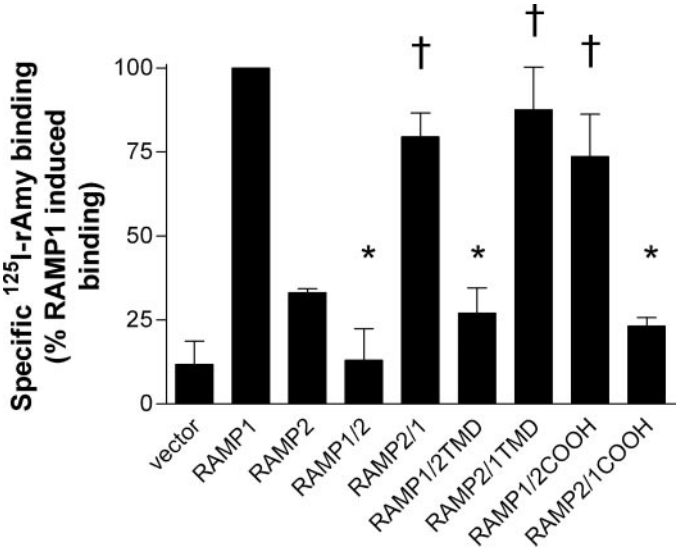

Fig. 2. Specific [^{125}I]rAMY binding to COS-7 cells coexpressing CTRa and wild-type RAMPs or RAMP domain swap chimeras, expressed as a percentage of RAMP1-induced binding. Experiments were carried out in 24-well plates using 100 ng/well receptor and 150 ng/well RAMPs. Total binding ranged from 2500 to 5000 cpm, and nonspecific binding ranged from 1000 to 2000 cpm for RAMP1-cotransfected cells. Data are mean ± S.E.M. of four separate experiments (*, $P < 0.05$ versus RAMP1-induced binding; †, $P < 0.05$ versus RAMP2-induced binding; paired t test).

Phenotype of AMY Receptors Induced by RAMP Domain Swap Chimeras. The pharmacological profile induced by wild-type and chimeric RAMPs was further explored in peptide competition for [^{125}I]AMY binding to COS-7 cells coexpressing CTRs and RAMPs. The RAMP1/CTRa and RAMP2/CTRa receptors had similar moderate to high-affinity for rAMY and sCT but differed in their affinity for hCGRP and hCT; the RAMP1/CTRa receptor having higher affinity for hCGRP than for hCT, whereas at the RAMP2/CTRa, the order of potency was reversed (Fig. 3, A and B; Table 1). This difference was used to compare the phenotypes induced by the domain swap chimeras. The chimera RAMP1/2COOH, containing only the C terminus of RAMP2, induced a phenotype very similar to that induced by wild-type RAMP1, in which the affinity for hCGRP was higher than for hCT (Fig. 3, A and C; Table 1). In contrast, the RAMP2/1TMD-induced phenotype was essentially equivalent to that induced by RAMP2 (Fig. 3, B and D; Table 1). [^{125}I]AMY binding to CTRa coexpressed with either of the chimeras RAMP1/2TMD or RAMP2/1COOH was low and inconsistent, and no reliable estimates of affinity were obtained. These data are consistent with the RAMP N-terminal domain playing the principal role in determining the modified binding affinity of interacting receptors.

The Role of RAMP Transmembrane and Carboxy-Terminal Domains in Ligand-Induced cAMP Responses with CTRs. The RAMP TMD and C terminus were also assessed for the effect on peptide-induced cAMP responses. In COS-7 cells, rAMY and hCGRP had low potency, and hCT and sCT had high potency at CTRa alone (Fig. 4 A and Table 2). When RAMP1 was coexpressed, the potency of hCGRP and rAMY increased, whereas RAMP2 coexpression induced only a weak increase in rAMY potency (Fig. 4, A versus B, and C; Table 2). The phenotype induced by chimera RAMP1/2TMD was similar to that of RAMP1 (Fig. 4D and Table 2). RAMP2/1TMD and RAMP1/2COOH, which contained the TMD of RAMP1 and thus exhibited high [^{125}I]rAMY binding, induced increased rAMY potency relative to the vector cotransfected cells; however, neither led to a change in hCGRP potency (Fig. 4, E and F; Table 2). The RAMP1/2COOH chimera also had a ~10-fold decrease in potency for hCT (Fig. 4E and Table 2). In contrast, the RAMP2/1COOH chimera induced a phenotype that was essentially equivalent to that of RAMP1 (Fig. 4, G versus B, and C; Table 2), implying that the C terminus of RAMPs affects signaling.

Discussion

RAMPs can be divided into three major structural domains: the N-terminal domain, the transmembrane domain, and the C-terminal domain. Evidence from early studies suggested that the extracellular N-terminal domain is important in determining ligand specificity. First, [^{125}I]CGRPα was shown to cross-link to RAMP1 and CLR, indicating close proximity of RAMP1 and CLR at the cell membrane (Stangl et al., 1991; McLatchie et al., 1998). Second, Fraser et al. (1999) showed that the chimera RAMP1/2, which contains only the N terminus of RAMP1, coexpressed with CLR in

human embryonic kidney 29T cells, revealed a CGRP receptor similar to that seen with RAMP1, whereas the reverse chimera (RAMP2/1) revealed an adrenomedullin receptor similar to that induced by RAMP2 when coexpressed with CLR, suggesting that the N-terminal domain was sufficient to engender specificity of peptide interaction. The RAMP1/2 chimera gave a significantly higher level of [^{125}I]adrenomedullin binding than wild-type RAMP1 (Fraser et al., 1999), suggesting that other regions of RAMP may be involved in determining the overall phenotype.

The current study demonstrates that each of the major functional RAMP domains can independently affect the resultant phenotype of RAMP/CTR dimers. In agreement with earlier data from N-terminal domain exchange chimeras (Zumpe et al., 2000), the presence of the RAMP1 N-terminal domain in individual domain swap chimeras led to equivalent specificity of peptide inhibition of [^{125}I]AMY binding to that seen with wild-type RAMP1, whereas those with the RAMP2 N terminus were equivalent to wild-type RAMP2. These profiles were seen regardless of the total level of [^{125}I]AMY binding, providing further evidence for the predominant role of the N terminus in determining peptide binding affinity; collectively, the data are also consistent with the direct involvement of the RAMP N terminus in the formation of the binding pocket for peptides acting at RAMP-based AMY, CGRP, and adrenomedullin receptors.

The separation of the transmembrane and C-terminal domains in the current study also allowed for investigation into the respective contribution of each of these domains to receptor function. Expression of CTRa and RAMP1 or RAMP2 in COS-7 cells reveals differences in the total level of [^{125}I]AMY binding induced (Christopoulos et al., 1999; Tilakaratne et al., 2000; Zumpe et al., 2000), allowing discrimination of the role that each of the domains plays in this behavior. Previous work in our laboratory established that the level of binding was principally influenced by the TMD/C terminus that was present rather than the N-terminal domain (Zumpe et al., 2000). The individual domain swap chimeras identified the TMD as the major determinant of the level of [^{125}I]AMY binding and therefore as likely to be an important contributor to the stability of RAMP/CTR complexes. Two recent articles have examined the role of RAMP1 domains in the formation of the CGRP receptor (Steiner et al., 2002; Fitzsimmons et al., 2003). Truncation of 20 amino acids from the RAMP1 TMD resulted in poor expression at cell surface and complete loss of CGRP-stimulated cAMP response in human

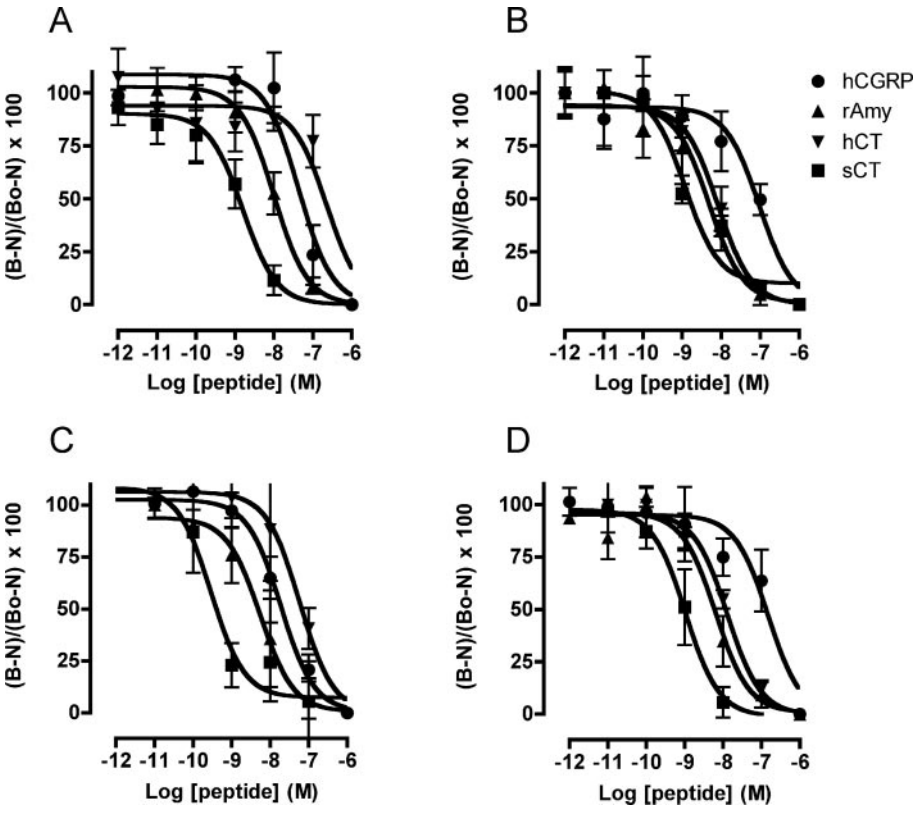

Fig. 3. Competition for [^{125}I]AMY binding to COS-7 cells cotransfected with hCTRa and RAMP1 (A), RAMP2 (B), RAMP1/2COOH (C), or RAMP2/1TMD (D) with hCGRPα (●), rAMY (▲), hCT (▼), or sCT (■). Data are mean ± S.E.M. of four or more separate experiments. B, [^{125}I]rAMY bound; B_0, total binding in the absence of competing peptide; N, nonspecific binding (measured in the presence of 10^{-6} M peptide). pIC$_{50}$ values are given in Table 1.

TABLE 1
pIC$_{50}$ values for peptides in competition for [^{125}I]rAmy binding to COS-7 cells cotransfected with hCTRa and WT or chimeric RAMPs
Data are presented as mean ± S.E.M. ($n \geq 4$).

	hCGRPα*	rAmy	hCT*	sCT
CTRa + RAMP1	7.36 ± 0.15	8.11 ± 0.13	6.66 ± 0.40	8.71 ± 0.16
CTRa + RAMP2	6.85 ± 0.30	8.29 ± 0.19	8.01 ± 0.18**	8.93 ± 0.17
CTRa + RAMP1/2COOH	7.53 ± 0.34	8.32 ± 0.25	7.29 ± 0.21	9.39 ± 0.30
CTRa + RAMP2/1TMD	6.55 ± 0.33**	8.20 ± 0.18	7.64 ± 0.21**	8.63 ± 0.18

*, significantly different between constructs (F test; $P < 0.05$).
**, significantly different from RAMP1 (unpaired t test; $P < 0.05$).

embryonic kidney 293 cells (Steiner et al., 2002). Using constructs of the RAMP1 N terminus fused with the TMD and C terminus of the platelet-derived growth factor receptor, or the RAMP N terminus alone, Fitzsimmons et al. (2003) showed that although the N terminus alone was sufficient for association and cell surface trafficking of CLR, this occurred with lower efficiency than with wild-type RAMP1. Furthermore, the loss of the RAMP1 TMD led to a decrease in CGRP affinity and potency for stimulation of cAMP production. This was possibly due to destabilization of the association between the RAMP1 constructs and CLR; evidenced by secretion of the RAMP1 N terminus into the media (Fitzsimmons et al., 2003). In both studies, the TMD was essential for normal CGRP receptor function and supported a role for the RAMP1 TMD in the stability of the RAMP/CLR complex. The results of the present study suggest that the RAMP TMD may play a similar role with CTR, although, like the interactions with CLR, the TMD is not the sole site of interaction between CTR and the RAMP, with a significant role likely to be played by other domains (M. Udawela and P. M. Sexton, unpublished data), as suggested by the differences in induction of AMY phenotype by RAMPs in different cellular backgrounds and across isoforms of the CTR that differ in intracellular domain 1 (Tilakaratne et al., 2000).

The role of RAMP domains in signaling of AMY receptors has essentially not been studied, and the current study represents the first to delineate the relative contributions of individual RAMP domains to signaling. The previous lack of investigation is due in part to the greater difficulty in the interpretation of signaling data that is caused by the background CTR-alone phenotype that is present in cotransfection experiments because of the efficient cell surface expression of CTRs, even in the absence of RAMPs (Christopoulos et al., 1999; Hay et al., 2005). In accord with previous studies, RAMP1 cotransfection with CTRa resulted in an increase in hCGRP and rAMY potency compared with CTRa alone, whereas RAMP2 cotransfection had little effect on receptor phenotype (Christopoulos et al., 1999; Muff et al., 1999; Hay et al., 2005). Chimeras containing the RAMP1 C terminus (RAMP1/2TMD and RAMP2/1COOH) had signaling profiles equivalent to that induced by RAMP1, whereas the potency of peptides for those constructs with the RAMP2 C terminus

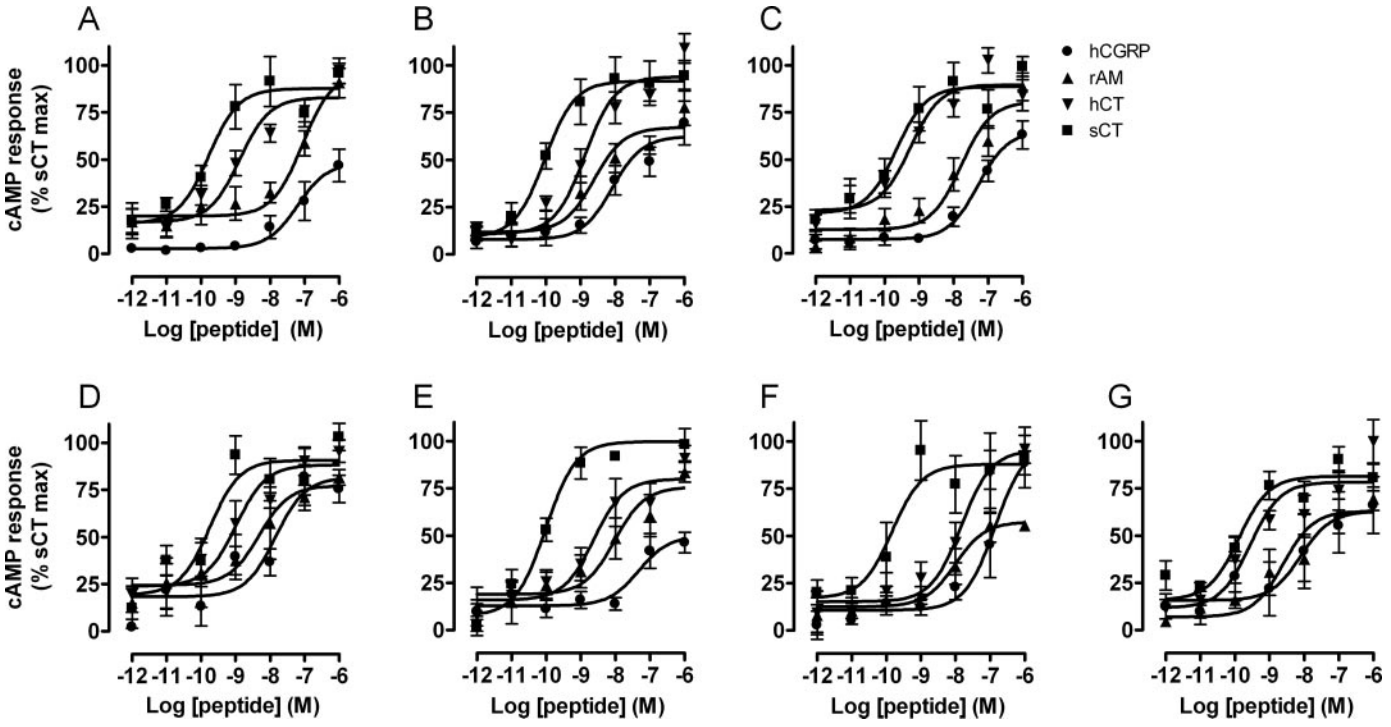

Fig. 4. Induction of cAMP accumulation in COS-7 cells cotransfected with hCTRa and empty vector (A), RAMP1 (B), RAMP2 (C), RAMP1/2TMD (D), RAMP2/1TMD (E), RAMP1/2COOH (F), or RAMP2/1COOH (G) by hCGRPα (●), rAMY (▲), hCT (▼), or sCT (■). Data are mean ± S.E.M. of four or more separate experiments, normalized to the maximal sCT response. $p\mathrm{EC}_{50}$ values are given in Table 2.

TABLE 2

$p\mathrm{EC}_{50}$ values for cAMP induction in COS-7 cells cotransfected with hCTRa and WT or chimeric RAMPs

Data are presented as mean ± S.E. ($n \geq 5$).

	hCGRPα*	rAmy*	hCT*	sCT
CTRa + vector	7.20 ± 0.27	7.06 ± 0.22	8.88 ± 0.18	9.78 ± 0.24
CTRa + RAMP1	8.08 ± 0.25**	8.61 ± 0.25**	8.87 ± 0.14	10.03 ± 0.19
CTRa + RAMP2	7.29 ± 0.20	7.78 ± 0.22**	9.25 ± 0.23	9.66 ± 0.28
CTRa + RAMP1/2TMD	7.84 ± 0.36	8.32 ± 0.45**	8.99 ± 0.26	9.75 ± 0.30
CTRa + RAMP2/1TMD	7.33 ± 0.37	8.02 ± 0.33**	8.63 ± 0.28	10.01 ± 0.17
CTRa + RAMP1/2COOH	6.86 ± 0.22	8.01 ± 0.31**	7.83 ± 0.28**	9.83 ± 0.41
CTRa + RAMP2/1COOH	8.09 ± 0.52	8.60 ± 0.31**	9.56 ± 0.31	9.87 ± 0.22

*, significantly different between constructs (F test; $P < 0.05$).
**, significantly different from vector control (unpaired t-test; $P < 0.05$).

(RAMP2/1TMD and RAMP1/2COOH) were similar to that seen with RAMP2. The minor exception to this was the RAMP1/2COOH construct in which, in addition to increased rAMY potency, there was a decrease in hCT potency. This latter behavior is consistent with the increase in [^{125}I]AMY binding seen with this construct, with the decrease in hCT potency also being indicative of reduced levels of free CTRa (Hay et al., 2005). Thus, despite little influence of the TMD/C terminus in specificity of peptide binding, the relative potency of peptides was differentially modified according to which C-terminal sequence was present. This was particularly true for hCGRP potency but was also seen to a lesser extent for rAMY, in which a weaker increase in potency was observed where the RAMP2 C terminus was present. These data are indicative of a critical role for the short C-terminal domain in the signaling by CTRa/RAMP-based AMY receptors, potentially via a role in G protein or regulatory protein interaction. This contrasts with the data for CLR-RAMP1-based CGRP receptors, in which the deletion of eight or nine amino acids of the RAMP1 C terminus had little effect on receptor signaling (Steiner et al., 2002; Fitzsimmons et al., 2003), possibly because of the importance of receptor component protein in signaling via CLR-based receptors (Evans et al., 2000), and suggests that the RAMP C terminus plays a more crucial role in phenotypic induction with CTRs.

In conclusion, the current work provides evidence for distinct contributions of each of the major RAMP domains in CTR-based AMY receptors: the N-terminal domain, playing a key role in defining the binding site for peptides; the transmembrane domain, in the stabilization of RAMP-receptor complexes; and the C-terminal domain, in the signaling profile of receptors. The data also highlight apparent differences between the behavior of CTR/RAMP and CLR/RAMP complexes that may be important in targeting receptor complexes for drug development.

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Address correspondence to: Dr. Patrick M. Sexton, Department of Pharmacology, Building 13E, Monash University, Clayton, 3800, Victoria, Australia. E-mail: patrick.sexton@med.monash.edu.au