Calcitonin and Amylin Receptor Peptide Interaction Mechanisms

INSIGHTS INTO PEPTIDE-BINDING MODES AND ALLOSTERIC MODULATION OF THE CALCITONIN RECEPTOR BY RECEPTOR ACTIVITY-MODIFYING PROTEINS

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Receptor activity-modifying proteins (RAMP1–3) determine the selectivity of the class B G protein-coupled calcitonin receptor (CTR) and the CTR-like receptor (CLR) for calcitonin (CT), amylin (Amy), calcitonin gene-related peptide (CGRP), and adrenomedullin (AM) peptides. RAMP1/2 alter CLR selectivity for CGRP/AM in part by RAMP1 Trp-84 or RAMP2 Glu-101 contacting the distinct CGRP/AM C-terminal residues. It is unclear whether RAMPs use a similar mechanism to modulate CTR affinity for CT and Amy, analogs of which are therapeutics for bone disorders and diabetes, respectively. Here, we reproduced the peptide selectivity of intact CTR, AMY1 (CTR-RAMP1), and AMY2 (CTR-RAMP2) receptors using purified CTR extracellular domain (ECD) and tethered RAMP1- and RAMP2-CTR ECD fusion proteins and antagonist peptides. All three proteins bind salmon calcitonin (sCT). Tethering RAMPs to CTR enhanced binding of rAmy, CGRP, and the AMY antagonist AC413. Peptide alanine-scanning mutagenesis and modeling of receptor-bound sCT and AC413 supported a shared non-helical CGRP-like conformation for their TN(T/V)G motif prior to the C terminus. After this motif, the peptides diverged; the sCT C-terminal Pro was crucial for receptor binding, whereas the AC413/rAmy C-terminal Tyr had little or no influence on binding. Accordingly, mutant RAMP1 W84A- and RAMP2 E101A-CTR ECD retained AC413/rAmy binding. ECD binding and cell-based signaling assays with antagonist sCT/AC413/rAmy variants with C-terminal residue swaps indicated that the C-terminal sCT/rAmy residue identity affects affinity more than selectivity. rAmy(8–37) Y37P exhibited enhanced antagonism of AMY1 while retaining selectivity. These results reveal unexpected differences in how RAMPs determine CTR and CLR peptide selectivity and support the hypothesis that RAMPs allosterically modulate CTR peptide affinity.

Receptor activity-modifying proteins are single-pass transmembrane proteins (RAMP1–3 in humans) that form heteromeric complexes with several G protein-coupled receptors (GPCRs) and thereby regulate their cell-surface expression and pharmacology (1, 2). RAMPs are best characterized for their effects on two class B GPCRs, the calcitonin receptor (CTR) and CTR-like receptor (CLR) (3). CTR/CLR and their complexes with RAMPs give rise to at least seven pharmacologically distinct receptors (not including splice variants) in humans that exhibit unique selectivity profiles for six related calcitonin (CT) family peptide agonists as follows: CT, amylin (Amy), calcitonin gene-related peptides α and β (αCGRP and βCGRP), adrenomedullin (AM), and adrenomedullin2/intermedin (AM2) (2–4). CLR-RAMP1 is the CGRP receptor at which CGRP has greater potency than AM. CLR-RAMP2 and CLR-RAMP3 are the AM1 and AM2 receptors, respectively, at which AM is more potent than CGRP (1). AM2 activates the CGRP and AM receptors, but is most potent at AM2 (5). CTR alone is the CT receptor at which CT is more potent than Amy. CTR complexes with RAMP1, -2, or -3 form distinct AMY1, AMY2, and AMY3 receptor subtypes, respectively (6–9), at which CT and Amy are roughly equipotent; the AMY1 and AMY3 receptors also respond to CGRP (9–11). This complex system provides a paradigm for understanding modulation of GPCRs by accessory proteins, but much remains unknown regarding the mechanisms of RAMP-altered peptide recognition.

Several of the diverse biological actions of CT family peptides are clinically relevant thus highlighting the need to understand peptide recognition mechanisms to facilitate drug development. CGRP and AM are potent vasodilators (12). Antagonism of the neurogenic inflammatory effects of CGRP is actively pursued as migraine therapy (13), and the cardioprotective effects of AM suggest that agonists of its receptor(s) may be of value for various cardiovascular disorders (14). The potent CT receptor agonist salmon calcitonin (sCT) has long been used to treat bone disorders taking advantage of its essentially irreversible
binding to the human receptor (15) and the ability of exogenous CT to affect bone turnover (16). Amy controls plasma glucose levels via AMY receptor activation that causes slowed gastric emptying, inhibition of glucagon secretion, and reduction of food intake (17). The Amy analog pramlintide is available for types I and II diabetes, and AMY receptor activation is under consideration as an obesity treatment (17, 18). Recently, amylin-mediated metabolic alteration was shown to induce regression of p53-deficient tumors (19). Despite these drug development successes and potential, relatively little is known about how CT and Amy bind their receptors, and next-generation analogs with more favorable properties are actively pursued (20, 21).

Peptide agonist binding to class B GPCRs follows a “two-domain” model (22). Binding of the peptide C-terminal region to the N-terminal extracellular domain (ECD) of the receptor contributes to affinity and selectivity and facilitates binding of the N-terminal peptide region to the receptor seven transmembrane domain (7-TM), which activates the receptor. N-terminally truncated agonist peptides thus act as antagonists. The two-domain model applies to the CT family peptides and their receptors, but with the additional complication of the RAMP subunits (23).

Our understanding of CT family peptide receptor binding is most advanced for CGRP and AM (24). Recombinant soluble ECDs of RAMP1 and RAMP2 formed complexes with the CLR ECD that bound their respective peptides, albeit with lower affinity than the full-length receptors (25–28). Although these complexes were stable enough to co-purify, we devised a fusion protein approach in which the C-terminal fragments of a CGRP analog or AM were tethered with a flexible (Gly-Ser)5 linker sequence. The following three plasmid constructions were introduced into RAMP1 (W84A) or RAMP2 (E101A) using the Gibson Assembly method. All plasmids were assembled into the pHLsec vector designed for secreted expression from mammalian cells (36) using PCR/restriction enzyme/DNA ligase-based cloning methods or the Gibson Assembly method using Gibson Assembly Master Mix (New England Bio-labs). Primer sequences are available upon request. The CTR fusion protein designs were similar to those previously reported for CLR (29). The RAMP and CTR ECDs were tethered with a flexible (Gly-Ser)3 linker sequence. The following three plasmids were constructed by inserting DNA encoding the desired MBP fusion constructs between the AgeI and KpnI sites of pHLsec as follows: pHLsec/MBP-hCTR.36–151-(His)6; pHLsec/MBP-hRAMP1.24–111-(Gly-Ser)3-hCTR.36–151-(His)6; and pHLsec/MBP-hRAMP2.55–140-(Gly-Ser)3-hCTR.36–151-(His)6 (amino acid numbers indicated). Amino acid substitutions were introduced into RAMP1 (W84A) or RAMP2 (E101A) using the Gibson Assembly method. All plasmids were confirmed by automated DNA sequencing of the coding regions performed by the University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research core facility. Purification of the plasmids for use in transient transfections used the Macherey-Nagel Midi kit or Qiagen Giga kit according to the manufacturer’s directions.

It is unclear to what extent the recent findings for the CGRP and AM receptors translate to CTR and AMY receptors. Chimeric receptor studies indicated that the RAMP ECDs dictated ligand selectivity at CTR (11, 35), but there are no reports of purification and characterization of the CTR ECD or its complexes with RAMP ECDs to confirm these findings. The stoichiometry of CTR–RAMP complexes has not been studied. There are well characterized differences between RAMP complexes with CTR and CLR. CLR is an obligate heterodimer; without RAMP subunits it cannot reach the cell surface (1). In contrast, CTR can traffic to the cell surface and function independently of RAMPs (3). The ligand selectivity patterns conferred by RAMPs at CLR and CTR are different. Whereas RAMP1 and RAMP2 swap the selectivity of CLR for CGRP and AM, their association with CTR has only minor effects on CT affinity and potency (9, 11). Moreover, RAMP1 and RAMP2 both confer enhanced affinity for the same ligand, Amy, at CTR despite their favoring binding of different peptides, CGRP or AM, to CLR (9). These properties hint that RAMPs may use different mechanisms to alter peptide selectivity at CLR and CTR.

Here, we purified CTR ECD and tethered RAMP1–CTR and RAMP2–CTR ECD fusion proteins and investigated their peptide interactions. Mutagenesis of peptide ligands and RAMP1/2 ECDs and computational modeling were employed to probe the conformations of receptor ECD-bound CT and Amy or Amy analogs, and selected results were extended to intact receptors via cell-based signaling assays. We also swapped the C-terminal Pro of CT for the C-terminal Tyr of Amy and vice versa to investigate the role(s) of peptide C-terminal residues in affinity and selectivity at CTR. Our results reveal significant differences in how RAMPs function at CTR and CLR and are consistent with the hypothesis that RAMPs modulate CTR peptide affinity by an allosteric mechanism.

Experimental Procedures

Plasmid Construction and Purification—Human CTRα isoform, RAMP1, and RAMP2 cDNAs were obtained from the cDNA resource center. Gene fragments encoding Escherichia coli maltose-binding protein (MBP) ending with an NAAAE linker sequence, CTR ECD, RAMP1 ECD, or RAMP2 ECD were assembled into the pHLsec vector designed for secreted expression from mammalian cells (36) using PCR/restriction enzyme/DNA ligase-based cloning methods or the Gibson Assembly method using Gibson Assembly Master Mix (New England Bio-labs). Primer sequences are available upon request. The CTR fusion protein designs were similar to those previously reported for CLR (29). The RAMP and CTR ECDs were tethered with a flexible (Gly-Ser)3 linker sequence. The following three plasmids were constructed by inserting DNA encoding the desired MBP fusion constructs between the AgeI and KpnI sites of pHLsec as follows: pHLsec/MBP-hCTR.36–151-(His)6; pHLsec/MBP-hRAMP1.24–111-(Gly-Ser)3-hCTR.36–151-(His)6; and pHLsec/MBP-hRAMP2.55–140-(Gly-Ser)3-hCTR.36–151-(His)6 (amino acid numbers indicated). Amino acid substitutions were introduced into RAMP1 (W84A) or RAMP2 (E101A) using the Gibson Assembly method. All plasmids were confirmed by automated DNA sequencing of the coding regions performed by the University of Oklahoma Health Sciences Center Laboratory for Molecular Biology and Cytometry Research core facility. Purification of the plasmids for use in transient transfections used the Macherey-Nagel Midi kit or Qiagen Giga kit according to the manufacturer’s directions.
Flow filtration using three Minimate™ TFF capsules (methyleneimine transfection reagent according to standard methods (36). All post-expression processing and purification steps were carried out at 4 °C. Cell culture media were collected 72 h after transfection and centrifuged to remove remaining cells, and the supernatant was filtered with a 0.22 µm filter (Corning) and dialyzed overnight against 4 liters of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM imidazole using 6–8-kDa molecular mass cutoff dialysis membrane. Because of poor expression, the MBP-RAMP1 (W84A)-CTR ECD protein required scale-up into six expanded surface area roller bottles (with 1700-cm² surface area for each, Corning) with 350 ml of culture volume and 500 µg of plasmid DNA per bottle. In addition, after transfection the temperature was lowered to 30 °C, and the culture media were harvested after 4 days. The media (~2.1 liters) were centrifuged to remove cells, and the supernatant was filtered (0.22 µm, Millipore), concentrated to ~150 ml by tangential flow filtration using three Minimate™ TFF capsules (molecular mass cutoff of 10 kDa) connected in parallel and the Pall Minimate™ TFF system, and finally dialyzed as above.

After dialysis, the proteins were purified by immobilized metal affinity and size exclusion chromatography using an AKTA purifier system (GE Healthcare). A 5-ml pre-packed nickel-chelating Sepharose column (GE Healthcare) was equilibrated in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, followed by sample loading and extensive washing in equilibration buffer before step elution with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 275 mM imidazole, 10% (v/v) glycerol. Peak fractions were pooled and spin-concentrated using Pierce® concentrators with 9-kDa molecular mass cutoff. The concentrated sample was loaded on a 320-ml bed volume Superdex 200 HR column (GE Healthcare) equilibrated in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol. Peak fractions were pooled and dialyzed overnight against 1 liter of storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50% (v/v) glycerol) and stored as aliquots at −80 °C. The courses of the purifications were monitored by SDS-PAGE and native-gel electrophoresis was performed as described previously (29). Protein concentrations were determined by UV absorbance at 280 nm using extinction coefficients calculated based on Tyr residues and stored as aliquots at −80 °C. For the sCT (22–32) Y22A mutant peptide that has no Tyr, the concentration was calculated assuming 80% peptide content. N-terminally biotinylated peptides used for binding studies were as follows: B-sCT (1–32)NH₂ (C1A7A), biotin-ASNLSTAVLGKLSEQLHKLQTYPTNTGSGTP-NH₂; B-AC413 (1–25)NH₂, biotin-GATAATQRLANFLVQLTYPRTNVNGANTY-NH₂. Cysteines at position 1 and 7 of biotinylated sCT were substituted with alanine because the disulfide bond is dispensable for ECD interactions, and its removal lowered the cost of the synthetic peptide. Biotinylated AC413 has a short linker GATA before its sequence to minimize interference of peptide immobilization on peptide-ECD interactions. All peptides used were C-terminally amidated unless otherwise noted. Full-length AC413 and AC187 were N-terminally acetylated, but all truncated AC413 peptides were non-acetylated. sCT (22–32)NH₂ and AC413(6–25)NH₂ were used as the parent sequences for alanine scan. Sequence alignment was generated with ClustalX 2.1 (37) and visualized with ESPript 3.0 using % equivalent algorithm.

**Alpha-LISA Luminescent Proximity Peptide Binding Assay**—Peptide binding to purified proteins was assessed with AlphaLisa technology (PerkinElmer Life Sciences). In this bead-based assay, N-terminally biotinylated peptide is attached to the surface of streptavidin-coated donor beads, and the receptor fusion protein is attached to the surface of MBP antibody-coated acceptor beads via its MBP tag. Peptide-receptor interaction brings the beads into proximity, which generates a luminescent signal. MBP-ECD protein and corresponding biotinylated peptide were incubated at multiple concentrations to examine the optimal condition for maximal binding signal. Binding to MBP-CTR ECD and MBP-RAMP1/2-CTR ECD was measured with B-sCT and B-AC413, respectively. Competitive binding assay was performed with the optimal concentrations of the MBP-ECD protein and the biotinylated peptide (MBP-CTR ECD at 150 nM with 150 nM B-sCT, MBP-RAMP1-CTR at 200 nM with 100 nM B-AC413, and MBP-RAMP2-CTR at 120 nM with 100 nM B-AC413). Competitive binding assay with the RAMP1 (W84A)-CTR and RAMP2 (E101A)-CTR proteins was performed under the same conditions used for wild-type ECDs. The beads (15 µg/ml each) were mixed with MBP-ECD protein and peptides in reaction buffer (50 mM MOPS, pH 7.4, 150 mM NaCl, 7 mg/ml fatty acid free BSA), then incubated in a dark room until the reaction mixture reached equilibrium (5 h). Order of addition affected the signal level. For MBP-CTR ECD the competitor peptides were combined with a master solution that contained the beads, receptor, and B-sCT, whereas for MBP-RAMP1/2-CTR ECD the competitor peptides and B-AC413 were pre-mixed and then combined with a master solution containing beads and receptor.

Luminescence was read in 384-well white opti-plates (Greiner bio-one) using a PolarStar Omega plate reader equipped with filters for Alpha-LISA (BMG Labtech, Germany). Luminescent counts from all samples were transformed to percentage of maximum defined by the maximal counts without competitive peptides or with competitive peptides used at the lowest concentration. Competitive binding curves were produced by nonlinear regression using the one site-Fit logIC₅₀.
Peptide Binding to CTR ECD and RAMP-CTR ECD Complexes

equation of Prism 5.0 (GraphPad Software). Means and S.E. of duplicate samples were shown in the binding curves, and the representative curves of at least three independent experiments were used for figures.

Saturation binding data were not fit by nonlinear regression because the two-bead assay format and consequent multivalent nature of the Alpha-LISA assay and the “hook effect” render standard binding equations inappropriate for this assay. For this reason also the IC_{50} values from competition assays were not converted to K_i values, although we have previously observed that under conditions similar to those used here IC_{50} values from the competition assays provide reasonable estimates of the true affinities (29). All competitor peptides were checked for nonspecific inhibition by testing their ability to inhibit the Alpha-LISA signal obtained with an MBP-RSPO2 fusion protein and biotinylated ZNRF3 ECD, which are unrelated to CTR and RAMP1-2-CTR complexes (38). None of the peptides exhibited significant inhibition in the control assay at the concentrations used here (data not shown).

cAMP Signaling Assay—COS-7 cells were transiently transfected by the reverse transfection method. For AMY_{1} expression, pcDNA3.1 constructs expressing full-length CTR and RAMP1 (each 50 ng/well) were mixed with FuGENE® HD transfection reagent (300 nl/well, Promega). After 5 min of incubation at room temperature, the mixture (5 |l|/well) was added to each well of a 96-well plate (Costar). Then COS-7 cells (ATCC) (2 × 10^5 cells in 100 |l|) were seeded to each well and incubated for 48 h with FuGENE® reagent and DNA constructs. For CTR expression, the DNA construct expressing CTR (50 ng/well) was combined with empty pcDNA3.1 vector (50 ng/well). After 48 h of incubation, the cells were washed with PBS twice and pre-incubated for 30 min with or without antagonist peptides in cAMP assay buffer (DMEM with 1 mM isobutylmethylxanthine and 0.1% fatty acid-free BSA). Then, the cells were treated with the hCT or rAmy agonist for 15 min in the presence or absence of the antagonist used for pre-incubation. After 15 min at 37 °C, 5% CO_2, the peptide solution was removed from cells by pipetting, and the cells were fixed with 100% cold ethanol (50 |l|/well) for 10 min at −20 °C, which was completely evaporated under a fume hood afterward (over 2 h). cAMP was extracted with cAMP detection buffer (50 mM HEPES, pH 7.4, 10 mM CaCl_2, 0.35% Triton X-100) and measured using a LANCE cAMP kit according to the manufacturer's instructions (PerkinElmer Life Sciences). A PolarStar Omega plate reader equipped with a time-resolved fluorescence advanced optic head (BMG Labtech, Germany) was used for LANCE TR-FRET detection. Concentration-response curves were generated by nonlinear regression using the log(agonist) versus response equation of Prism 5.0 (GraphPad software). Means and S.E. of triplicate or duplicate samples were shown for concentration-response curves, and the representative curves of at least three independent experiments were used for figures.

pA_2 Calculation for Antagonist Potency—pA_2 indicates the negative logarithm of the antagonist concentration that right-shifts the agonist concentration-response curve by 2-fold as a measure of antagonist affinity. We chose 50% of response for pA_2 calculation and used the Gaddum/Schild EC_{50} shift equation with Hill and Schild slopes constrained to 1 in Prism 5.0 (GraphPad Software). Concentration-response curves of cAMP production mediated by agonist alone or in the presence of the antagonist were used to calculate pA_2. Means ± S.E. are shown in Table 3.

Statistical Analysis—For the binding experiments, pIC_{50} converted from IC_{50} was used for statistical analysis. Statistical differences in pIC_{50} and pA_2 were analyzed with one-way analysis of variance using Tukey’s post hoc test or Student’s t test (for two groups) using Prism 5.0 (GraphPad Software).

Homology Modeling—Homology models of the RAMP1-CTR ECD complex bound with sCT(22–32) or AC413(15–25) were constructed using Swiss-Model (39) in DeepView/Swiss-PdbViewer (version 4.1.0) with the RAMP1-CLR ECD fusion protein bound with a CGRP analog as a template (Protein Data Bank code 4RWG). Side chain rotamers of CTR Glu-123 and Asn-124 in the homology models were manually adjusted to reduce steric clashes with the peptide ligands. PyMOL (Schrodinger) was used for graphical illustration.

Results

Production of Recombinant Soluble CTR ECD and Tethered RAMP1/2 ECD-CTR ECD as MBP Fusion Proteins—We sought to produce highly purified soluble CTR ECD and CTR-RAMP ECD complexes for structure/function studies. To this end we employed the MBP-tethered ECD fusion protein approach previously developed for CLR-RAMP ECD complexes (29). Three expression constructs were generated that encode human CTR and CTR-RAMP complexes engineered as shown in Fig. 1A. The ECD(s) are linked to MBP, which can serve as a “crystallization module” for future structural studies (24). The CTR and RAMP ECDs were tethered by a flexible (Gly-Ser)_{5} linker, which ensures stability of the complexes in the absence of the membrane-embedded portions of the receptors and enforces 1:1 CTR-RAMP stoichiometry. We chose the eukaryotic HEK293T cell expression system for producing the engineered constructs as secreted proteins because we had trouble producing them in E. coli (data not shown), and N-glycosylation of CTR has been reported to influence its ligand binding (40). Each purified protein yielded a sharp and symmetrical peak on gel filtration at the elution volume corresponding to the monomer (Fig. 1B and data not shown). Non-reducing SDS-PAGE and non-denaturing native PAGE demonstrated the high purity of the final samples, although they exhibited some heterogeneity that was likely due to differential N-glycosylation (Fig. 1C).

Calcitonin Family Peptide Binding Profiles of Purified CTR ECD and Tethered RAMP1/2-CTR ECD Fusion Proteins—Binding of synthetic calcitonin family peptides to the recombinant fusion proteins was assessed with Alpha-LISA luminescent proximity assay technology (PerkinElmer Life Sciences). The sequences of the peptides relevant to the binding studies are shown in Fig. 2. Experiments with the MBP-CTR ECD protein used biotinylated salmon CT (B-sCT) as the probe, whereas studies with the MBP-RAMP1/2-CTR ECD proteins used biotinylated AC413 (B-AC413), which served as a putative AMY mimic because we could not detect binding with biotinylated rat Amy (rAmy) (data not shown). AC413 is an AMY receptor antagonist that has significant sequence similarity to...
Peptide Binding to CTR ECD and RAMP-CTR ECD Complexes

A) 

|       | MBP CTR ECD | MBP RAMP1 ECD | MBP RAMP2 ECD |
|-------|-------------|---------------|---------------|
| N     |             |               |               |
|       |             |               |               |
|       | 36          | 151           | 36            |
|       | 24          | 111           | 36            |
|       |             |               | 24            |
|       |             | 55            | 36            |
|       | 140         | 36            |               |
|       | 151         | 36            |               |

B) 

|       | CTR | RAMP1-CTR | RAMP2-CTR |
|-------|-----|-----------|-----------|
| mAU   |     |           |           |
| 0     | 50  | 100       | 150       |
| 50    | 100 | 150       | 200       |
| 100   | 150 | 200       | 250       |
| 150   | 200 | 250       | 300       |

C) 

SDS-PAGE (non-reducing)  
Tris-Glycine native gel

FIGURE 1. Expression and purification of CTR ECD and RAMP1/2-CTR ECD fusion proteins in HEK293T cells. A, protein constructs designed for this study. Five pairs of (Gly-Ser) were used as a linker between RAMP1/2 and CTR ECDs. Amino acid numbers used for the ECDs are indicated above the diagram. The schematic shown is not scaled with real protein size. B, gel filtration elution profiles of MBP-CTR ECD, MBP-RAMP1-CTR ECD, and MBP-RAMP2-CTR ECD fusion proteins. C, left image is non-reducing SDS-PAGE analysis. Molecular mass markers are shown in the 1st lane and are labeled in kDa. Tris-glycine native gel analysis is shown in the right panel where a slight upward shift was observed for the E101A mutant protein due to the loss of a negative charge on Glu-101. The gels were stained with Coomassie Brilliant Blue.

Amy (41). Competition assays using non-biotinylated peptides enabled determination of apparent affinities (IC_{50} values). N-terminally truncated antagonist fragments of sCT, human calcitonin (hCT), rAmy, hαCGRP, and human AM were used as competitors. Binding of AMY receptor antagonist peptides AC413 and AC187 (42) and an antagonist fragment (residues 8–37) of the diabetes drug pramlintide (43) were also evaluated. AC413 and AC187 (42) and an antagonist fragment (residues 8–37) of the diabetes drug pramlintide (43) were also evaluated. AC413 and AC187 (42) and an antagonist fragment (residues 8–37) of the diabetes drug pramlintide (43) were also evaluated.

The effects of the sCT Y22A and P32A mutations varied for the three ECD proteins. Tyr-22 contributed to binding CTR ECD and RAMP2-CTR ECD, but not RAMP1-CTR ECD, whereas Pro-23 contributed to binding RAMP1-CTR ECD but not the other two ECD proteins (Fig. 4, A–C). Full competitive binding curves for these mutants indicated that Y22A decreased the pIC_{50} of sCT(22–32) at CTR ECD, although P32A failed to change pIC_{50} (Table 1). At RAMP1-CTR ECD, Y22A was unable to change sCT(22–32) binding, but P32A showed a decrease in pIC_{50}. The effects of Y22A and P32A at RAMP2-CTR ECD mimicked the pattern at CTR ECD. These results indicated that RAMP1 and RAMP2 have subtly different effects.
Nonetheless, overall the alanine scan data and modeling suggested that sCT(22–32) binds CTR ECD and RAMP1/2-CTR ECD fusion proteins in a similar manner and with a CGRP-like conformation.

**Identification of a Minimal ECD-binding AC413 Fragment**—
Truncation of the N-terminal five amino acids did not change the pIC50 of AC413 at CTR ECD and RAMP1/2-CTR ECD, but extending the truncation to 8–11 amino acids showed significant decreases in pIC50 at the three ECD proteins with a greater decrease by the truncation of 11 amino acids (Table 1). Truncation of 17 amino acids abolished binding to the three ECD proteins. These results indicated that the minimal AC413 fragment that retains the same ECD-binding affinity as the full-length peptide is an 18–20-mer, which is almost two times longer than the minimal sCT(22–32) fragment and thus indicated differences in how the two peptides contact the receptors.

**Identification of Critical Residues of AC413 for CTR ECD and RAMP1/2-CTR ECD Binding**—The minimal AC413(6–25) fragment was selected for alanine-scanning mutagenesis targeting all non-alanine residues. To discern effects of mutations at all three independent experiments are shown except huGGRP(8–37) with CTR and AM(22–52) with CTR, RAMP1-CTR, and RAMP2-CTR (n = 2). The means of duplicate samples with S.E. are shown in the binding curves.

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**Identification of Critical Residues of AC413 for CTR ECD and RAMP1/2-CTR ECD Binding**—The minimal AC413(6–25) fragment was selected for alanine-scanning mutagenesis targeting all non-alanine residues. To discern effects of mutations at all three proteins, mutant AC413(6–25) peptides at single concentrations of 100 and 10 μM were used for competition binding to CTR ECD and RAMP1/2-CTR ECD fusion proteins, respectively. T18A and V20A dramatically decreased the binding of AC413(6–25) to CTR ECD (Fig. 5A). F8A, L9A, L12A, Y15A, R17A, N19A, and N23A mutations moderately decreased CTR ECD binding. These effects were repeated at RAMP1-CTR and RAMP2-CTR (Fig. 5, B and C). AC413(6–25) G21A was insoluble at 100 μM rendering it unsuitable for competition binding with CTR ECD, but 10 μM G21A mutant was soluble and greatly decreased binding to RAMP1/2-CTR ECD (Fig. 5, B and C). The Y25A mutation showed a difference for the three ECD proteins; it slightly decreased binding to RAMP1-CTR but...
Peptide Binding to CTR ECD and RAMP-CTR ECD Complexes

| Peptide                  | CTR ECD Mean ± S.E. | RAMP1-CTR ECD Mean ± S.E. | RAMP2-CTR ECD Mean ± S.E. |
|-------------------------|---------------------|---------------------------|---------------------------|
| hCTR(8–32)              | 3.96 ± 0.07         | ND                        | ND                        |
| hCTR(8–32)              | 2.01 ± 0.03         | 3.67 ± 0.07               | 3.38 ± 0.04               |
| hCTR(8–32)              | 3.73 ± 0.05         | 3.78 ± 0.08               | 3.48 ± 0.04               |
| sCTR(8–32)              | 10.65 ± 0.04        | 5.65 ± 0.03               | 5.57 ± 0.03               |
| sCTR(8–32)              | 5.62 ± 0.06         | 10.59 ± 0.03              | 7.45 ± 0.02               |
| sCTR(8–32)              | 4.66 ± 0.06         | 3.98 ± 0.15               | 4.35 ± 0.09               |
| sCTR(8–32)              | 3.82 ± 0.04         | 5.49 ± 0.06               | 6.62 ± 0.05               |
| sCTR(8–32)              | 5.41 ± 0.03         | 5.07 ± 0.02               | 5.16 ± 0.06               |
| sCTR(8–32)              | 3.09 ± 0.07         | 4.15 ± 0.06               | 4.24 ± 0.02               |
| AC413(6–25)             | 4.51 ± 0.04         | 5.66 ± 0.07               | 6.57 ± 0.08               |
| AC413(6–25)             | 4.64 ± 0.01         | 5.71 ± 0.05               | 5.80 ± 0.03               |
| AC413(1–25)             | 4.29 ± 0.01         | 4.38 ± 0.01               | 4.95 ± 0.10               |
| AC413(1–25)             | 4.09 ± 0.03         | 4.54 ± 0.03               | 4.56 ± 0.03               |
| AC413(15–15)            | 4.18 ± 0.03         | 4.55 ± 0.06               | 4.60 ± 0.03               |
| AC413(18–25)            | 2.01 ± 0.03         | 2.01 ± 0.03               | 2.01 ± 0.03               |
| AC413(25–25)            | 4.60 ± 0.05         | 5.18 ± 0.06               | 5.65 ± 0.02               |
| AC413(6–25)             | 5.80 ± 0.05         | 5.80 ± 0.05               | 5.80 ± 0.05               |
| AC413(6–25)             | 6.26 ± 0.02         | 6.79 ± 0.05               | 7.12 ± 0.04               |
| rAmy(8–37)              | 3.93 ± 0.04         | 4.36 ± 0.05               | 4.24 ± 0.05               |
| rAmy(8–37)              | 3.93 ± 0.04         | 4.26 ± 0.05               | 4.02 ± 0.08               |
| rAmy(8–37)              | 3.97 ± 0.01         | 5.43 ± 0.01               | 5.67 ± 0.02               |

* p < 0.05 as compared with sCTR(22–32).

* p < 0.05 as compared with AC413(6–25).

* p < 0.05 as compared with rAmy(8–37). NS, no significance compared with the template peptide used for mutagenesis. Analysis of variance with Tukey’s post hoc test or Student’s t test (for two groups) was used for statistical analysis.

The Y25A mutation of the C-terminal residues of sCT, CGRP, and AM, which dramatically diminished receptor ECD binding (Fig. 4), (28, 29, 44). We were unable to assess the effects of the R11A and P16A substitutions because of peptide solubility issues.

The 11-mer AC413(15–25) fragment, which is similar in sequence to CGRP (Fig. 2), was modeled bound to RAMP1-CTR ECD based on the CGRP(27–37) analog-bound RAMP1-CLR ECD crystal structure (Fig. 5D). The alanine-scanning data agreed well with part of the model; Thr-18 hydrogen bonds with CTR Asp-101, Thr-18 and Val-20 contact a hydrophobic patch in CTR ECD, and Gly-21 facilitates turn formation, consistent with the importance of these residues indicated by the binding results. However, the model failed to explain the minimal effect of the C-terminal Y25A mutation because Tyr-25 was predicted to occupy the pocket over Trp-79 thereby enabling contact between the Tyr-25 phenyl ring and RAMP1 Trp-84.

To further analyze the AC413 C-terminal residue, we performed full competition binding assays with the Y25A mutant. Consistent with Fig. 5, the Y25A mutation decreased the pIC50 value of AC413 to only a small degree at RAMP1-CTR and had no effect on the pIC50 value at CTR ECD or RAMP2-CTR (Fig. 6A; Table 1). AC413(6–25) Y25F exhibited wild-type binding indicating that the aromatic ring of Tyr-25 is involved in RAMP1-CTR interaction, whereas the hydroxyl group is dispensable. We also assessed the role of the C-terminal Tyr-37 residue of rAmy(8–37), although the weaker affinity of rAmy(8–37) for the ECD proteins made it difficult to unambiguously determine the effects of mutations. We did not detect binding of rAmy(8–37) Y37A to CTR ECD or RAMP1-CTR ECD at the maximum 100 μM concentration tested, but it appeared to bind RAMP2-CTR ECD similar to wild type (Fig. 6B). Overall, the binding studies and modeling suggested that the mid-portion of AC413 (residues 15–21) binds CTR ECD and RAMP1/2-CTR ECD complexes with a CGRP-like conformation and indicated that the C terminus of AC413, and perhaps that of rAmy, functions differently than its counterparts in sCT, CGRP, and AM.

RAMP1 Trp-84 and RAMP2 Glu-101 Do Not Significantly Contribute to Peptide Binding to RAMP-CTR ECD Fusion Proteins—In the crystal structures of CGRP analog-bound RAMP1-CLR and AM-bound RAMP2-CLR ECD, the phenyl ring of CGRP analog Phe-37 makes a hydrophobic contact with RAMP1 Trp-84 and the AM Tyr-52 hydroxyl group forms a key hydrogen bond with RAMP2 Glu-101 (Fig. 7A). These contacts “anchor” the peptides and contribute to affinity and selectivity (24). One hypothesis for how RAMP1/2 enhance affinity for Amy at CTR is via a direct peptide contact mechanism in which RAMP1 Trp-84 or RAMP2 Glu-101 contacts the Amy Tyr-37 phenyl ring or hydroxyl group, respectively. The alanine-scanning data presented above argue against this for RAMP2, but the small defect of AC413 Y25A and apparent defect of rAmy Y37A for RAMP1-CTR binding (Fig. 6) may result from the loss of contact with RAMP1 Trp-84. To assess the function of RAMP1 Trp-84 and RAMP2 Glu-101 in RAMP-CTR complexes, we expressed and purified RAMP1- and RAMP2-CTR ECD containing the RAMP1 W84A or RAMP2 E101A mutation (Fig. 1C) and tested their peptide binding.

The RAMP1/2 mutations had no detectable effect on B-AC413 binding to RAMP-CTR fusion proteins in a saturation binding assay format (Fig. 7, B and C). Competition binding assays produced pIC50 for sCT(8–32), AC413(6–25), and rAmy(8–37) that were comparable with those observed with wild-type RAMP-CTR fusion proteins (Fig. 7, B and C; Table 2). For reasons that are unclear, the CGRP(8–37) peptide yielded a competition curve that did not fit well with fixed slope (Hill slope = 1) at RAMP1 W84A-CTR ECD (Fig. 7B); thus, we cannot rule out a slight change of CGRP in binding this mutant protein. Nonetheless, these results clearly indicated that RAMP1 Trp-84 and RAMP2 Glu-101 do not play a significant role in peptide binding to RAMP-CTR complexes, which stands in contrast to their important roles in RAMP-CLR complexes.

Probing Affinity and Selectivity Determinants by Reciprocal Exchange of the C-terminal Residues of sCT and AC413/rAmy—Given that the sCT and AC413/rAmy binding studies presented thus far suggested different roles for their C-terminal residues, we asked what effect reciprocal exchange of these residues would have on affinity/selectivity for CTR ECD and RAMP1/2-CTR ECD. The P32Y swap in sCT(22–32) significantly decreased binding to all three ECD proteins (Fig. 8A), consistent with the importance of sCT Pro-32. In contrast, the Y25P swap in AC413(6–25) remarkably increased its binding to CTR ECD and RAMP1/2-CTR ECD fusion proteins with more than 1 log order increase in IC50 (Fig. 8A; Table 1). The rAmy(8–37) Y37P swap mutant also had significantly increased affinity for CTR ECD and RAMP1/2-CTR ECD fusion proteins (Fig. 8B; Table 1).

We performed cAMP signaling assays in COS-7 cells transiently expressing the CTR or AM receptors to test the antag-
onist activity of the swap mutants at the intact receptors. The C-terminal alanine substitution mutants were also included for comparison. Using hCT as an agonist, we observed significantly enhanced antagonism of cAMP production at the CTR by the AC413(6–25) Y25P swap mutant as compared with wild-type AC413(6–25) as predicted by the ECD binding data (Fig. 9A; Table 3). The Y25P swap mutant also exhibited increased ability to antagonize rAmy-mediated cAMP production at the AMY1 receptor, but the effect was not as dramatic as at CTR (Fig. 9B; Table 3). When hCT was used as the agonist at AMY1, the Y25P swap mutant exhibited increased antagonism similar to that observed at CTR alone (Fig. 9C; Table 3). Notably, AC413(6–25) Y25P was no longer selective for AMY1 over CTR (Table 3), although it appeared to retain moderate selectivity in the ECD binding assay (Fig. 8A; Table 1). The Y25A substitution in AC413(6–25) resulted in decreased antagonism of rAmy-mediated AMY1 activation as compared with the wild-type peptide (Fig. 9D; Table 3), but it had no effect on antagonism of CTR or AMY1 when hCT was the agonist (Fig. 9, A and C; Table 3). These results are consistent with the ECD binding data (Fig. 6A).

The rAmy(8–37) Y37P swap mutant exhibited antagonism significantly greater than the wild-type peptide at CTR (Fig. 9D; Table 3) and AMY1, regardless of the agonist used for activation (Fig. 9, E and F; Table 3). These results were in good agreement with the ECD binding data (Fig. 8B). Notably, in contrast to the AC413(6–25) Y25P swap, the rAmy(8–37) Y37P swap retained selectivity for AMY1 over CTR (pA2 of 7.52 for AMY1 and 6.73 for CTR; Table 3). rAmy(8–37) Y37A had no defect in its ability to antagonize rAmy-mediated activation of AMY1 (Fig. 9E; Table 3), which indicated that Tyr-37 is dispensable for binding the intact RAMP1-CTR complex in cells.

Discussion

Extracellular Domains of the CTR, AMY1, and AMY2 Receptors Selectively Bind CT Family Peptides—In this study we demonstrated for the first time, to our knowledge, that soluble monomeric CTR ECD and heterodimeric RAMP1/2-CTR ECD complexes, produced as monomeric tethered fusions enforcing 1:1 stoichiometry, reproduce the peptide selectivity profiles of the intact CTR, AMY1, and AMY2. Our results strongly suggest that the AMY1 and AMY2 receptors function as 1:1 heterodimers at least with respect to peptide binding. Notably, the tethered ECD approach greatly facilitated our ability to study the AMY receptor ECD complexes because MBP-CTR ECD-H6 and RAMP1 ECD-H6 co-expressed in HEK293T cells could be co-purified via their His tags, but they did not co-elute in subsequent gel filtration chromatography (data not shown), suggesting that the RAMP1 ECD complex with CTR is less stable than that with CLR.
The rank order of peptide binding affinity at CTR ECD was $sCT(8–32) > hCT(8–32)$. Tethering of RAMP1 or -2 ECD to CTR ECD enhanced the binding of Amy and Amy-like peptides giving a rank order of $sCT(8–32) > AC413 > hoCGRP(8–37) > rAmy(8–37)$, which is consistent with the intact AMY1 receptor (10, 11). The IC$_{50}$ values observed for $sCT(8–32)$ were similar at the three ECD proteins as observed for the intact receptors (10). The binding data thus indicated that the ECD constructs reproduced the selective peptide binding properties of the intact receptors. One caveat is that the binding affinity of AC187 was lower compared with AC413 both at CTR ECD and RAMP1/2-CTR ECD, although it has been characterized as a high affinity antagonist to intact CTR and AMY receptors with similar ability to AC413 (10). Differential contacts between the peptides and the transmembrane domains of CTR and/or RAMP likely explain this discrepancy.

We used purified N-glycosylated CTR ECD in our binding experiments as opposed to the non-glycosylated protein produced in E. coli used for several other class B GPCR ECD studies (24, 30–32, 34, 45, 46). The apparent affinities of the N-glycosylated CTR ECD proteins for their respective peptide ligands were in the micromolar range, which is weaker than the intact receptors, but consistent with observations for other E. coli-produced non-glycosylated class B GPCR ECDs. Thus, it is likely that the micromolar peptide affinities reported for other class B GPCR ECDs are not due to a lack of $N$-glycans but rather due to a lack of peptide contacts to the receptor 7-TM domain. However, whether or not CTR $N$-glycans influence ligand binding affinity and potency as reported in full-length receptors (40) remains an open question.

There is limited understanding of AMY2 due to conflicting reports and its apparent cell type dependence (7–9). RAMP2 co-expression with CTR in COS-7 cells failed to induce amylin receptor phenotype (7, 10) and showed much weaker binding of rAmy than RAMP1 expression (35). Significantly less binding of rAmy with RAMP2 expression was also observed in transfected rabbit aortic endothelial cells (8). However, Tilakaratne et al. (9) reported hCTRI (the same as CTRA isoform) expression in CHO cell induced amylin binding with all three RAMPs. Morfis et al. (47) reported equal binding affinity of rAmy to AMY1, AMY2, and AMY3 transfected into COS-7 cells, while showing much weaker potency of rAmy at AMY2 versus AMY1 and AMY3. Our results with RAMP2-CTR ECD support the role of RAMP2 in conferring increased Amy affinity at CTR.

The binding of alanine-substituted AC413(6–25) peptides to CTR ECD and RAMP1/2-CTR ECD fusion proteins and homology model of the AC413-bound RAMP1-CTR ECD complex. AC413(6–25) peptides with an alanine mutation were used at 100 and 10 μM concentrations for CTR ECD (A) and RAMP1/2-CTR ECD fusion proteins (B and C) binding, respectively. R11A and P16A mutant peptides were excluded for all ECD binding, and G21A mutant peptide was excluded only for CTR binding due to peptide insolubility. The concentrations of MBP-ECD proteins and biotinylated peptides used for the binding assay were the same as shown in Fig. 3. The averages of two independent experiments with duplicate samples are shown with S.E. in the bar graphs. The mutational effects on peptide binding are indicated with dark gray color, and stronger effects are indicated with additional slash lines. D, homology model of AC413(15–25) bound to the RAMP1-CTR ECD complex. The hydrophobic patch and binding pocket are labeled with A and B, respectively. The hydrogen bond of AC413(15–25) Thr-18 and CTR Asp-101 is shown as a red dotted line.
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sCT(22–32) May Adopt a Non-helical CGRP-like Receptor-bound Conformation—Alanine-scanning mutagenesis of sCT(22–32) identified Thr-25, Thr-27, Gly-28, and C-terminal Pro-32 as key residues for binding to CTR and RAMP1/2-CTR fusion proteins (Fig. 4). Alanine substitutions of hCGRP Thr-25, Val-20, and Gly-21 are critical for RAMP1/2-CTR ECD interactions consistent with the homology model (Fig. 5). The conservation of the TNVG motif in AC413, rAmy, and CGRP (Fig. 2) supports the modeling and suggests that rAmy(27–37) likely also adopts a non-helical CGRP-like conformation for the region comprising this motif. Indeed, NMR structures of amylin showed a highly flexible and unstructured C-terminal portion (51, 52).

Mutagenesis of the C-terminal region of sCT to the TN(T/V)G motif revealed significant differences among sCT/CGRP and AC413/rAmy. Whereas the C-terminal residues of sCT, CGRP, and also AM were critical for receptor ECD binding (Fig. 4) (28, 29, 44), the C-terminal AC413 Tyr-25 was dispensable for binding CTR and RAMP2-CTR and only minimally influenced binding to RAMP1-CTR (Fig. 6A). rAmy Y37A had no effect at RAMP2-CTR ECD but did appear to diminish RAMP1-CTR ECD binding. However, the low affinity of rAmy for RAMP1-CTR ECD prevented us from clearly determining the effects of the Y37A substitution (Fig. 6B). More importantly, this result failed to hold up in the cell-based cAMP signaling experiments where rAmy(8–37) Tyr-37 was clearly dispensable for antagonism of AMY1 (Fig. 9E).

The peptide mutagenesis data seem to suggest that the C-terminal residue of AC413 does not occupy the putative CTR pocket. Instead, it may be directed slightly away from the pocket. Notably, mutating the penultimate sCT residue Thr-31 diminished sCT binding, whereas mutating the corresponding AC413 Thr-24 had no effect on its binding. This suggests that the regions of these peptides subsequent to the putative turn-favoring glycine residue (sCT Gly-28 or AC413 Gly-21) either have different conformations or that they occupy different molecular environments. We attempted to further probe the role of the C terminus of AC413 by examining the ability of the free acid form of AC413(6–25) that lacks the amide group to bind the receptors (data not shown), but technical issues with this peptide prevented us from definitively assessing the role of the C-terminal amide. The Amy C-terminal amide was previously shown to be important for bioactivity (53).

An additional complication for AC413 was the role of N-terminal residues 8–12 in ECD binding, and modeling did not account for this. This portion of the peptide would be predicted to project “down” from the ECD toward the 7-TM bundle in the intact receptor and is unlikely to contact the RAMP. This region probably contacts the C and/or N termini of the CTR ECD. In the homology models (Figs. 4 and 5), the C terminus of the CTR ECD stops at residue 135 even though the construct used for the binding assays extends to residue 151, so there are extra C-terminal CTR residues missing from the models that may provide contacts to the N-terminal region of AC413. The
extra affinity conferred by AC413 residues Phe-8, Leu-9, Val-10, and Leu-12 probably makes up for the lack of receptor contacts by its Thr-24 and Tyr-25 residues thereby enabling equal affinity to sCT at RAMP1/2-CTR.

**RAMPs Function Differently at CTR and CLR**—Although RAMP1 Trp-84 and RAMP2 Glu-101 directly contact the C-terminal residues of CGRP and AM in the CGRP and AM1 receptors, the pIC\textsubscript{50} values of peptide ligands at the RAMP-CTR ECD fusion proteins with the RAMP1 W84A and RAMP2 E101A mutations were comparable with those for the wild-type tethered ECDs (Fig. 7, B and C). These data indicated that these RAMP residues are not required for function at CTR and thereby revealed significant differences between RAMP/CLR complexes. The simplest explanation for this difference is that the C-terminal Tyr of AC413/Thr-122 backbone of CLR ECD are shown as red dotted lines. B and C, saturation binding of B-AC413 with RAMP1 W84A-CTR ECD fusion protein and RAMP2 E101A-CTR ECD fusion protein on the left and their competitive binding on the right. 100 nM mutant ECD fusion proteins were used for saturation binding assay. Competitive binding assay was performed under the same concentrations as shown in Fig. 3. Representative saturation and competitive binding curves of three independent experiments are shown. The means of the duplicate samples and S.E. are shown in the binding curves.

**TABLE 2**

| Peptide | RAMP1-CTR W84A | RAMP2-CTR E101A |
|---------|----------------|-----------------|
| n | Mean | S.E. | n | Mean | S.E. |
| hCGRP(8–37) | 3 | 5.06 | 0.10 | 3 | 4.95 | 0.08 |
| sCT(8–32) | 3 | 5.51 | 0.05 | 4 | 5.94 | 0.02 |
| AC413(6–25) | 3 | 5.48 | 0.01 | 3 | 5.79 | 0.13 |
| rAmy(8–37) | 3 | 4.26 | 0.05 | 3 | 4.18 | 0.18 |

**FIGURE 7.** Effects of RAMP1 W84A and RAMP2 E101A on peptide binding to RAMP1/2-CTR ECD fusion proteins. A, superposition of crystal structures of CGRP analog-bound RAMP1-CLR ECD (Protein Data Bank code 4RWG) and AM-bound RAMP2-CLR ECD (Protein Data Bank code 4RWF) highlighting the pocket occupied by the C-terminal residue of the peptides. The hydrogen bonds between AM Tyr-52 and RAMP2 Glu-101 and between C-terminal amides of AM and CGRP analog and Thr-122 backbone of CLR ECD are shown as red dotted lines. B and C, saturation binding of B-AC413 with RAMP1 W84A-CTR ECD fusion protein and RAMP2 E101A-CTR ECD fusion protein on the left and their competitive binding on the right. 100 nM mutant ECD fusion proteins were used for saturation binding assay. Competitive binding assay was performed under the same concentrations as shown in Fig. 3. Representative saturation and competitive binding curves of three independent experiments are shown. The means of the duplicate samples and S.E. are shown in the binding curves.

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Peptide Binding to CTR ECD and RAMP-CTR ECD Complexes

FIGURE 8. Effects of C-terminal swap mutations of sCT(22–32) and AC413(6–25)/rAmy(8–37) on CTR ECD and RAMP1/2-CTR ECD fusion protein binding. A, sCT(22–32)/P32Y/AC413(6–25)/Y25P; B, rAmy(8–37)/Y37P binding to CTR ECD and RAMP1/2-CTR ECD fusion proteins. The concentrations of MBP-ECT proteins and biotinylated peptides used for the binding assay were the same as shown in Fig. 3. Representative competitive binding curves of three independent experiments are shown. The means of duplicate samples and S.E. are shown in the binding curves.

rAmy C-terminal Tyr residue in the ECD binding and cell-based antagonism experiments and why the lack of RAMP1 Trp-84 or RAMP2 Glu-101 side chain is of no consequence.

An alternative possibility is that RAMP1 Trp-84 and RAMP2 Glu-101 do not augment the putative pocket of CTR in the same way as they do for CLR and therefore they do not contact the peptides. The CLR and CTR ECD sequences are 64% identical. There are only six differences near the peptide-binding site, and most of these are at the periphery of the site; thus, the core peptide-binding site appears to be largely conserved in CLR and CTR. In contrast, the CTR ECD N-terminal α-helix predicted as the interface with RAMPs contains several residues, including Asp-50, Lys-54, Asp-57, and Gln-60 that are different from the corresponding CLR residues Thr-43, Glu-47, Gln-50, and Met-53. These differences might cause different positioning of RAMP1 Trp-84 and RAMP2 Glu-101 in complexes with CTR as compared with CLR.

The reality may be a complex combination of altered positioning of RAMP1 Trp-84 and RAMP2 Glu-101 in complexes with CTR as well as the C terminus of Amy and Amy-like peptides not occupying the putative pocket in the same manner as CGRP and AM. The slight defect in binding of AC413 Y25A to the RAMP1-CTR ECD (Fig. 6A) that translated to decreased antagonism of the intact AMY1 receptor (Fig. 9B) suggests contact between this residue and the receptor. This contact might involve the putative pocket in such a manner that Tyr-25 does not touch RAMP1 Trp-84.

Our results with the RAMP1 W84A ECD protein contrast a report that found diminished signaling potency of rAmy agonist at intact AMY1 containing RAMP1 W84A (54). This result was interpreted as indicating an important role for RAMP1 Trp-84 at AMY1. One possible explanation for the apparent discrepancy is that the truncated antagonist peptides we used bind slightly differently than full-length agonist peptides. Alternatively, the effect of RAMP1 W84A on agonist potency at AMY1 may have been due to the reported lower cell surface expression of AMY1-RAMP1 W84A. The ECD protein containing RAMP1 W84A also expressed very poorly, which suggests a folding/assembly defect for RAMP1 W84A in the context of the AMY1 receptor. Notably, the apparent defect resulting from W84A in intact AMY1 was not as dramatic as its effect in the CGRP receptor (55).

Role of the sCT/AC413/rAmy C-terminal Residues in Receptor Affinity and Selectivity—The properties of the sCT/AC413/ rAmy C-terminal residue swaps and alanine substitutions provide insights into the role(s) of the C-terminal residues in determining receptor affinity and selectivity. The increased ECD binding affinities of AC413(6–25)/Y25P and rAmy(8–37)/Y37P translated to increased antagonism of the intact CTR and AMY1 receptors in COS-7 cells, albeit less dramatically for AC413(6–25)/Y25P at AMY1 with rAmy as the agonist. The AC413 Y25P swap lost selectivity for AMY1 over CTR in the antagonism assay, whereas the rAmy(8–37)/Y37P swap retained selectivity (Fig. 9, D and E; Table 3). The differences observed for the AC413 and rAmy peptides indicate that AC413 is not an ideal Amy mimic. Although it shares considerable sequence identity with Amy, there are significant differences, including the lack of the central NLGPV segment and other substitutions (Fig. 2). The results for the more physiologically relevant rAmy(8–37)/Y37P swap mutant indicated that the identity of the C-terminal Amy residue dictates affinity more than selectivity. Moreover, the antagonism assay with rAmy(8–37)/Y37A clearly indicated no role for the Amy C-terminal Tyr side chain in contacting the receptor. These results starkly contrast the key functions of the C-terminal residues of CGRP and AM in receptor binding and selectivity (24) and highlight diversity in the mechanisms used by CT family peptides to bind their respective receptors.

We observed differences in the abilities of the peptides to antagonize AMY1 depending on the agonist used to activate the receptor (Fig. 9, B, C, E, and F). This agonist-dependent antagonism was previously reported and suggested to result either from overlapping but non-equivalent binding sites for CT and Amy peptides or from complications resulting from the two receptor populations, free CTR and AMY1, that likely exist in co-expression experiments (10). Our results for AMY1 with hCT as the agonist mirrored those obtained for CTR alone with hCT as the agonist. It would thus appear that the experiments in Fig. 9, C and E, primarily probed CTR occupancy.

The ECD binding and cell-based antagonism data for the C-terminal swap mutants and alanine substitutions were gen-
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FIGURE 9. Antagonism of AC413(6–25) Y25P/Y25A and rAmy(8–37) Y37P/Y37A at the CTR and AMY1 receptors in COS-7 cells. A, AC413(6–25) antagonism against hCT-mediated cAMP production at CTR. B, AC413(6–25) antagonism against rAmy-mediated cAMP production at AMY1. C, AC413(6–25) antagonism against hCT-mediated cAMP production at AMY1. D, rAmy(8–37) antagonism against hCT-mediated cAMP production at CTR. E, rAmy(8–37) antagonism against rAmy-mediated cAMP production at AMY1. F, rAmy(8–37) antagonism against hCT-mediated cAMP production at AMY1. Concentration-response curves of cAMP production were generated by nonlinear regression using the log(agonist) versus response equation of Prism 5.0. Representatives of at least three independent experiments are shown. The means of duplicate or triplicate samples and S.E. are shown in the cAMP production curves. See Table 3 for pA2 values for the antagonists.

TABLE 3

| Peptide          | CTR with hCT agonist | AMY1 with rAmy agonist | AMY1 with hCT agonist |
|------------------|----------------------|------------------------|-----------------------|
|                  | n  | Mean | S.E. | n  | Mean | S.E. | n  | Mean | S.E. |
| AC413(6–25)      | 3  | 7.04 | 0.03 | 7  | 7.43 | 0.09 | 4  | 7.19 | 0.11 |
| AC413(6–25) Y25P | 3  | 8.48 | 0.01 | 4  | 8.03 | 0.08 | 4  | 8.71 | 0.11 |
| AC413(6–25) Y25A | 3  | 7.16 | 0.16 | 4  | 6.53 | 0.08 | 3  | 7.31 | 0.15 |
| rAmy(8–37)       | 3  | <5.2 | 3.58 | 0.09 | 3   | <5.2 |
| rAmy(8–37) Y37P  | 3  | 6.73 | 0.02 | 3  | 7.52 | 0.11 | 3  | 6.83 | 0.10 |
| rAmy(8–37) Y37A  | 3  | 5.33 | 0.11 | 3  | 6.02 | 0.03 | 3  | 5.46 | 0.14 |

\[a \ p < 0.05 \text{ compared with AC413(6–25).} \]

\[b \ p < 0.05 \text{ compared with rAmy(8–37).} \]

Generally in good agreement, but there were a couple of minor discrepancies. The increase in antagonism of AC413(6–25) Y25P at AMY1 with rAmy as the agonist in the cAMP assay was not as dramatic as what was expected from ECD binding, and rAmy(8–37) Y37A had no defect in antagonizing AMY1 with rAmy as the agonist in the cAMP assay although it did appear to have a defect in ECD binding. The basis for these discrepancies is unclear at this time. Although the ECD proteins may not entirely explain the peptide interactions with intact receptors, overall our data suggested that the engineered tethered constructs were not significantly altered in their properties compared with the intact receptors. Indeed, using these ECD proteins enabled us to identify rAmy(8–37) Y37P as an antagonist with comparable potency and selectivity to the commonly used AMY antagonists AC187 and AC413. This novel AMY antagonist may be an additional pharmacological tool of use in physiological studies of AMY receptors, although it is not an ideal AMY-selective antagonist because it was less than 10-fold selective over CTR. We expect that the tethered fusion proteins will facilitate future structure/function studies.

Allosteric Role for RAMPs in Modulating CTR Peptide Affinity—We propose that our results support the hypothesis that RAMPs act allosterically to modulate CTR conformation to determine its ligand affinity/selectivity. Our data taken as a whole imply that there are no direct contacts between Amy residue side chains and the RAMP subunits, at least for the ECD complexes. The mutagenesis and modeling data for the TN(T/V)G motif were consistent with Amy binding to the peptide-binding site on CTR analogous to that on CLR. In this case, only the C terminus of the peptide would be expected to be in proximity to the RAMP subunit and our cell-based antagonism data clearly indicated a lack of involvement of the C-terminal Amy Tyr residue in receptor contact. Although we cannot rule out the possibility of RAMP contacts to the Amy main chain, our data with the rAmy(8–37) Y37P swap peptide support an allo-
teric mechanism. This peptide retained selectivity for \(\text{AMY}_1\) over CTR despite being mutated at its C terminus to be like CT, which binds CTR in the absence of RAMPs. This would appear to suggest that the putative allosteric effects of RAMPs on CTR mediate binding of regions of Amy prior to the C-terminal residue. Interestingly, our results provide evidence for subtly different effects of RAMP1 and RAMP2 on CTR conformation, e.g. the small but significant differences in the sCT Y22A or P23A mutational effects on RAMP1-CTR versus RAMP2-CTR (Table 1). We suggest that although RAMP1 and RAMP2 have subtly different effects on CTR conformation, they cause some similar change in CTR conformation that yields enhanced ability to bind Amy. Elucidating the nature of these proposed conformational changes and the conformations of the last few residues of receptor-bound sCT/AC413/Amy will require high resolution crystal structures of ligand-bound CTR and RAMP-CTR ECD complexes. Fortunately, the tethered ECD fusion proteins developed herein should facilitate progress on the structural front.

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