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Multiple Ramp Domains Are Required for Generation of Amylin Receptor Phenotype from the Calcitonin Receptor Gene Product

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Calcitonin (CT), calcitonin gene-related peptide (CGRP), amylin, and adrenomedullin constitute a family of structurally related peptides that signal via either the calcitonin receptor-like receptor or the CT receptor, with receptor phenotype determined by coexpression of one of the three receptor activity-modifying proteins (RAMPs). The nature of the interaction between the receptor and RAMP was investigated using chimeras between RAMP1 and RAMP2 where the amino-terminal domain of RAMP1 was attached to the transmembrane domain and carboxy terminus of RAMP2 and called RAMP1/2, and vice versa for RAMP2/1. Cotransfection of wild-type or chimeric RAMPs with the insert-negative isoform of the human CT receptor ($hCTR_{I1-}$) into COS-7 cells resulted in the expression of ^{125}I-rat amylin binding sites. Highest specific binding was observed when either RAMP1 or RAMP2/1 were cotransfected, indicating the importance of the RAMP transmembrane domain and/or carboxy terminus for the degree to which amylin receptors are expressed. In contrast, the phenotype generated was primarily determined by the amino terminus, with similar RAMP1- and RAMP1/2-induced receptor phenotypes that had higher affinity for human CGRPα and lower affinity for human calcitonin than the RAMP2- and RAMP2/1-induced receptors. © 2000 Academic Press

Calcitonin (CT), calcitonin gene related peptide (CGRP), amylin and adrenomedullin constitute a family of structurally related peptides with distinct biological actions. They share a region of predicted α-helical secondary structure from amino acid residues 8–18, an invariant carboxy terminal aromatic residue and a disulfide bonded amino terminal loop of six to seven amino acids that are important for agonist activity (1–3). Each of these peptides has distinct high-affinity receptors distinguished by unique profiles of peptide-receptor interaction (1–5).

Accumulating evidence indicates that two Class II, seven transmembrane domain G-protein coupled receptors, the CT receptor-like receptor (CRLR) and the CT receptor form the basis of all the receptors for the CT/CGRP/amylin/adrenomedullin family of peptides (4, 5). Calcitonin receptors have been cloned from a number of different species and classified according to their high affinity and specificity for CT peptides (1, 4). The CRLR, named as such because of its significant amino acid sequence homology with the CT receptor was isolated by molecular cloning strategies (6, 7). However, in early analysis, this receptor failed to respond to any of the CT family of peptides (6) and therefore was deemed an orphan receptor. It is now clear that CRLR indeed plays a part in forming the receptor for CGRP or adrenomedullin, but only when associated with one of three recently discovered proteins termed receptor activity modifying proteins (RAMPs). RAMP1 trafficked CRLR forms a CGRP receptor, while RAMP2 or RAMP3 trafficked receptor forms an adrenomedullin receptor (8–10). Similarly, RAMPs can interact with the CT receptor gene product to induce expression of distinct amylin receptor phenotypes (11, 12). Co-transfection of RAMP1 with the most common variant of the human CT receptor (termed $hCTR_{I1-}$ or hCTR2) induced amylin receptors that have moderate to high affinity for salmon CT, amylin and CGRP but poor affinity for human CT, while RAMP3 induced receptors have poor affinity for CGRP. In contrast, RAMP2 caused only weak induction of amylin binding and the induced phenotype has yet to be characterized (11, 12).

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In this study we have analyzed the RAMP2 induced phenotype and used the differences between RAMP1 and RAMP2 induced amylin receptors to explore the mechanistic basis for RAMP-CT receptor interaction. Using chimeras of RAMP1 and RAMP2, where the amino terminal extracellular domains were exchanged, we have demonstrated the amino terminus and the transmembrane domain/intracellular C-terminus both play important and distinct roles in the induction of amylin receptor phenotypes.

MATERIALS AND METHODS

Salmon CT (sCT), human CGRPα, rat amylin, and human CT (hCT) were obtained from Peninsula Laboratories (Belmont, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (Hepes), gentamycin, Geneticin (G418), and Lipofectamine were from GibcoBRL, Life Technologies (Grand Island, NY), as were tissue culture plates and flasks. Bovine serum albumin (BSA) was obtained from Commonwealth Serum Laboratories (Parkville, Australia). *N*-succinimidyl 3-(4-hydroxy,5,-[^{125}I] *iodophenyl*) propionate (Bolton–Hunter reagent; 2000 Ci/mmol) was from Amersham (Buckinghamshire, UK). ^{125}I-rat amylin (specific activity 2000 Ci/mmol) was iodinated by the Bolton–Hunter method and purified by reverse-phase high-performance liquid chromatography as previously described (13). All other chemicals were from Sigma chemical company (St. Louis, MO) and were reagent grade or better.

The chimeric RAMP constructs were those described by Fraser *et al.* (9). The "splice" between RAMP1 and RAMP2 sequences coincides with the conserved Asp Pro Pro sequence at the "outside" of the transmembrane domain.

Cell culture. Green monkey kidney COS-7 cells were maintained in 150-cm^2 flasks at 37°C in a humidified atmosphere with 5% CO_2:95% air, in DMEM supplemented with 5% FBS, 80 mg/liter gentamycin, 1 mg/liter minocycline, and 15 mM Hepes. Cells were subcultured weekly using 0.025% trypsin in phosphate-buffered saline (PBS) (140 mM NaCl, 2 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.3) with 5 mM EDTA to harvest the cells.

Cell transfections. The amount of RAMP DNA to use in transfections was determined from preliminary experiments cotransfecting 100 ng/well $hCTR_{I1-}$ and increasing concentrations of RAMPs. RAMP (100 ng/well) was chosen for further transfections as it provided the highest level of induced amylin phenotype (not shown). For analysis of RAMP chimeras, COS-7 cells were cotransfected with Lipofectamine as previously described (11) with 100 ng of $hCTR_{I1-}$ subcloned into pZem228CC (14) and either pcDNA3 (Invitrogen, CA) vector alone or RAMP1, RAMP2, RAMP1/2 or RAMP2/1 subcloned into the pcDNA3 mammalian expression vector (8, 9). Cells were subcultured into 24 well plates and incubated for 24 h to grow cells to ~60% confluency. Complexes of $hCTR_{I1-}$ and RAMP DNA with Lipofectamine were formed by incubation of Lipofectamine and DNA in serum and antibiotic-free DMEM for 45 min at 22°C. The media in the wells was replaced with media containing DNA–Lipofectamine complexes and incubated for ~16 h prior to the addition of DMEM supplemented with 10% FBS. Cells were incubated for a further 24 h before competition binding assays were performed.

Receptor binding assays. Growth media was aspirated and binding buffer (DMEM with 0.1% BSA) containing ~100 pM ^{125}I-rat amylin was added in the absence or presence (10^{-11} to 10^{-6} M peptide) of competing unlabeled peptides rat amylin, human CGRPα, hCT or sCT. Non specific binding was defined as binding in the presence of 10^{-6} M unlabeled amylin. Cells were incubated at 37°C for 60 min, then binding buffer aspirated and unbound radioactivity washed from the wells with 0.5 ml ice-cold PBS. Cells were solubilized by the addition of 0.5 ml of 0.5 M NaOH and radioactivity was detected by a Packard gamma-counter (~75% efficiency). At least four experiments with triplicate repeats were performed for each radioligand binding experiment.

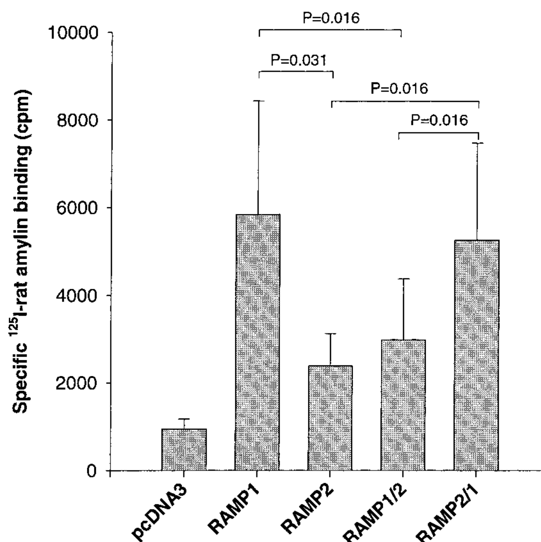


FIG. 1. Specific ^{125}I-rat amylin binding to COS-7 cells co-transfected with 100 ng $hCTR_{I1-}$ and 100 ng RAMPs or vector (pcDNA3) control. The level of RAMP induced binding was compared using Wilcoxon signed ranks tests where $P < 0.05$ was considered significant. Data are means ± SEM ($n = 7$).

Statistical analysis. Results of binding studies are shown as means ± SEM. For competition binding studies, 95% confidence limits and half maximal inhibitory concentrations (IC_{50}) values were calculated by non-linear regression using GraphPad PRISM (Version 3) (Graphpad Software, CA). Binding data were analyzed simultaneously for one site and two site fits, with the best-fit equation determined by an *F* test. Differences in binding levels were compared by a Wilcoxon signed ranks test (SigmaStat Version 2; SPSS Software, Inc., IL).

RESULTS

Both RAMP1 and RAMP2, co-transfected with $hCTR_{I1-}$ into COS-7 cells, increased the level of specific ^{125}I-rat amylin binding compared to the pcDNA3 control (Fig. 1). However, the magnitude of the increase in binding differed between the RAMP1 and RAMP2 induced receptors with RAMP1 generating higher levels of binding than RAMP2 ($P < 0.05$; $n = 7$, Wilcoxon signed ranks test) (Fig. 1).

Analysis of receptor phenotype by competition of ^{125}I-rat amylin binding with unlabeled peptides revealed that the receptor phenotypes generated by the two RAMPs were different. Each receptor phenotype had equivalent affinity for sCT and rat amylin, however the RAMP1 induced receptors had significantly higher affinity for CGRP and lower affinity for hCT than the RAMP2 phenotype (Table 1; Figs. 2A and 2B).

To further analyze the mechanism of RAMP-receptor interaction, chimeras between RAMP1 and RAMP2

TABLE 1

pIC_{50} Values (M) for Peptides in Competition for ^{125}I-Rat Amylin Binding to COS-7 Cells Cotransfected with $hCTR_{I1-}$ (100 ng) and Wild-Type or Chimeric RAMPs (100 ng) ($n = 4$)

		Rat amylin	Human CGRPα	Human calcitonin	Salmon calcitonin
RAMP1	Site 1	8.27 ± 0.11	7.87 ± 0.09	6.92 ± 0.10	9.05 ± 0.08
RAMP2	Site 1	7.82 ± 0.15	5.86 ± 0.23*	8.08 ± 0.29 (73%)***	8.89 ± 0.15
	Site 2			4.4 ± 10 (27%)	
RAMP1/2	Site 1	8.26 ± 0.17	7.71 ± 0.14	7.41 ± 0.15	9.12 ± 0.15
RAMP2/1	Site 1	8.14 ± 0.07	6.97 ± 0.17**	8.59 ± 0.24 (64%)***	9.01 ± 0.13
	Site 2			6.14 ± 0.35 (36%)	

Note. There is no significant difference in the receptor phenotype for RAMP1 and RAMP1/2. RAMP2 and RAMP2/1 phenotypes are equivalent except for a lower affinity for CGRP at RAMP2-induced receptors. RAMP2 has higher affinity for hCT and lower affinity for CGRP when compared with RAMP1.

* Lower affinity than RAMP1, RAMP1/2, and RAMP2/1 ($P < 0.05$).

** Lower affinity than RAMP1 and RAMP1/2 ($P < 0.05$).

*** RAMP2 and RAMP2/1 exhibit a two-site fit for hCT (F test, $P < 0.05$).

were studied. These chimeras were generated by splicing the amino terminus of RAMP1 with the transmembrane and carboxy terminal domains of RAMP2 (RAMP1/2), and vice versa (RAMP2/1) (9). Following co-transfection into COS-7 cells with $hCTR_{I1-}$, the level of ^{125}I-rat amylin binding induced by the RAMP1/2 chimera was equivalent to that of RAMP2, while the RAMP2/1 chimera induced similar binding levels to RAMP1 (Fig. 1). Thus the level of amylin binding induced by the RAMPs appears to be principally influenced by the transmembrane and carboxy terminal domains of RAMP.

In contrast, the RAMP1/2 induced amylin receptor phenotype was similar to that induced by RAMP1 with comparable affinities of the individual competing peptides for these sites (Table 1, Figs. 2A and 2C). Peptide binding at the RAMP2 and RAMP2/1 induced amylin receptors was also similar, except that human CGRPα had a slightly lower affinity at the RAMP2-derived receptor than at the RAMP2/1 induced receptor (Table 1, Figs. 2B and 2D). Rat amylin and sCT had the same affinity for each RAMP induced receptor phenotype (Table 1, Fig. 2). These data confirm that the differences in binding levels produced by co-expression of RAMPs and their chimeras reflect changes in receptor numbers and not their affinity for amylin.

DISCUSSION

RAMPs are integral membrane proteins with a large extracellular amino terminus, a single transmembrane spanning domain and a short (~10 amino acid) C-terminal intracellular tail. These proteins alter the phenotype of at least two G-protein coupled receptors; the CT receptor and CRLR (8, 11, 12), although, their mechanism of action is only partially understood. CRLR plus native RAMP1 or RAMP2 produces similar levels of two very different receptors, those for CGRP or adrenomedullin, respectively (8, 9). In contrast, RAMP1 and RAMP2, in this study, both induced ^{125}I-rat amylin binding when co-transfected with $hCTR_{I1-}$ into COS-7 cells, however, the level of induced binding and resultant receptor phenotypes differed significantly. These differences enabled the definition of functionally important domains for RAMP function through exchange of the amino terminal domains of RAMP1 and RAMP2.

The RAMP1-induced amylin receptor was equivalent to previously described RAMP1/$hCTR_{I1-}$-induced receptors (11, 12). In contrast, the RAMP2/$hCTR_{I1-}$-induced amylin receptor phenotype had not previously been characterized, as the level of RAMP2 induced binding in earlier studies too low to allow phenotypic analysis (11, 12). The RAMP2/$hCTR_{I1-}$ phenotype differed from the RAMP1/$hCTR_{I1-}$ phenotype in that it had higher affinity for hCT and lower affinity for CGRPα. Nonetheless, both receptors displayed equivalent affinity for rat amylin and sCT, indicating that the difference in the level of ^{125}I-amylin binding is due to differences in the capacity of RAMP1 and RAMP2 to interact with the CT receptor to alter phenotype.

Using the chimeric RAMPs to define the relative contribution of the amino terminal domain versus the transmembrane domain and carboxy terminus, we demonstrated that RAMP2/1 induced equivalent levels of amylin binding to that induced by RAMP1. Similarly the level of binding following RAMP1/2 cotransfection was equivalent to RAMP2 and was less than that induced by RAMP1 or RAMP2/1. As the affinity of amylin was equivalent for each RAMP-induced receptor the data indicates that the transmembrane domain and carboxy terminus of RAMP are important in defining the receptors' expression. This was not the case when the same chimeras were co-expressed with CRLR; RAMP1 and RAMP1/2 produced similar levels of CGRP binding. RAMP2 and RAMP2/1 produced similar levels

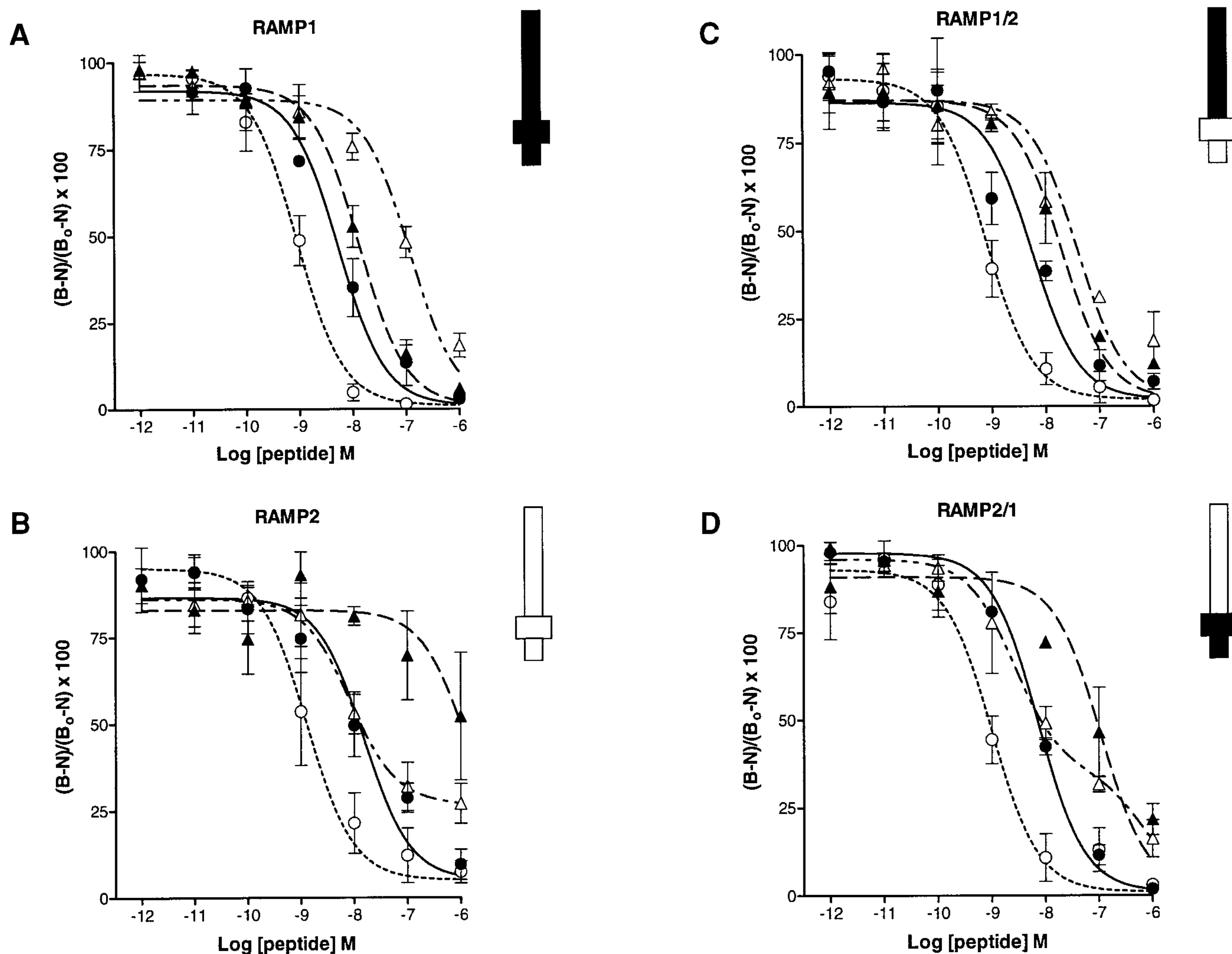
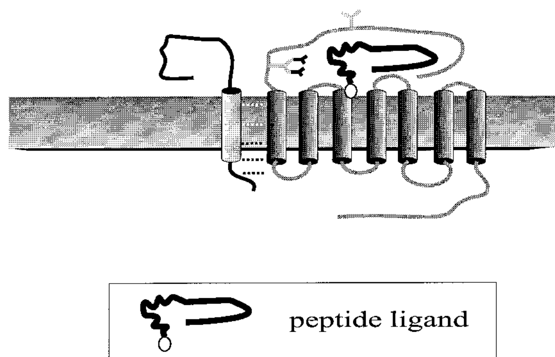


FIG. 2. Peptide competition for ^{125}I-rat amylin binding to COS-7 cells cotransfected with 100 ng $hCTR_{I1-}$ and 100 ng (A) RAMP1 (filled schematic), (B) RAMP2 (unfilled schematic), (C) RAMP1/2 or (D) RAMP2/1. Unlabeled rat amylin (●), sCT (○), hCT (△) and human CGRPα (▲) were used to compete for ^{125}I-rat amylin binding. Data are means ± SEM of four individual experiments each performed with triplicate repeats and are expressed as a percentage of specific amylin binding in the absence of competing peptide. B, ^{125}I-amylin bound; Bo, ^{125}I-amylin bound in the absence of competing peptide; N, nonspecific binding determined in the presence of 10^{-6} M unlabeled amylin. pIC_{50} values for these experiments are shown in Table 1.

of adrenomedullin binding. The appearance of CGRP or adrenomedullin binding correlated with the glycosylation state of CRLR, where "terminal" glycosylation was associated with the CGRP receptor phenotype (9). These data could be explained if the transmembrane domain and carboxy terminus of RAMP1 were able to form a tighter interaction with the CT receptor than could the corresponding domains of RAMP2. A more stable complex could lead to a greater level of expressed amylin receptor phenotype (Fig. 3). That the level of CGRP binding seen following RAMP1 and RAMP1/2 co-transfection with CRLR is equivalent may imply that the interaction between RAMP1 and RAMP2 with CRLR through the transmembrane domain and carboxy terminus is similar. Alternatively it may be that interaction between CRLR and RAMPs occurs principally through the amino terminal domain, in contrast to the likely mechanism of RAMP/CT receptor interaction.

The receptor phenotypes generated by co-transfection of RAMPs and $hCTR_{I1-}$ differed in their specificity of interaction for human calcitonin and human CGRPα. The RAMP1/2 induced phenotype was equivalent to the RAMP1 induced phenotype despite lower levels of expressed binding. The RAMP2 and RAMP2/1 binding sites also share a similar phenotype, with higher affinity for human calcitonin and lower affinity for human CGRPα compared with the RAMP1 and RAMP1/2 phenotypes. Thus, the receptor specificity for competing peptides appears to be primarily dictated by the amino terminus present, possibly a result of a direct contribution

A Principal interaction of RAMP and CT receptor occurs via the transmembrane domain/C-terminus



B Direct or allosteric contribution of RAMP amino terminus to the peptide binding pocket

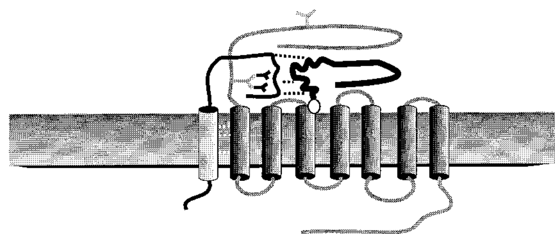


FIG. 3. Schematic representation of probable key interactions of RAMP and CT receptors. (A) The principal site of high affinity interaction between RAMP and the receptor is the transmembrane domain/C-terminus of RAMP and it is therefore the strength of this interaction that determines the level of expressed receptor phenotype. Following this the amino terminal domain of RAMP is able to modify the peptide-binding pocket of the receptor, either directly or indirectly (B), leading to induction of amylin receptor phenotypes. A minor role for the transmembrane domain/C-terminus in defining receptor phenotype, through either allosteric modulation of the receptor or effects on coupling proteins is also possible.

by the RAMP amino terminus to peptide binding pocket (Fig. 3).

This study provides insight into the molecular nature of RAMP-CT receptor interaction with the carboxy terminal and/or transmembrane domains of RAMP important for the level of induced receptor expression and the amino-terminal domain being the principal determinant of phenotype.

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