Distinct receptor activity modifying protein (RAMP) domains differentially modulate interaction with calcitonin receptors

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Running title:  Distinct roles for RAMP domains in receptor function

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Non standard abbreviations:  AMY, amylin receptor phenotype; CGRP, calcitonin gene-related peptide; CLR, calcitonin receptor-like receptor; CT, calcitonin; CTR, calcitonin receptor; GPCR, G protein-coupled receptor; HA, haemagglutinin epitope tag; hCT, human calcitonin; RAMP, receptor activity modifying protein; rAmy, rat amylin; sCT, salmon calcitonin; TMD, transmembrane domain.
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 formation of AMY receptors is poorly understood. We have used chimeras between RAMP1 and RAMP2 that specifically exchanged the N-terminal, transmembrane (TM) or C-terminal domain and examined these in assays of $^{125}$I-amylin binding or peptide induced cAMP signalling, 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 distinction between the transmembrane domain and C-terminus in this function. In contrast to the effects on binding, each of the RAMP domains played a role in the signalling phenotype of the receptors. In particular, the potency of calcitonin gene-related peptide (CGRP) was most influenced by the C-terminal domain present, where presence of the RAMP1 C-terminal domain led to increased potency over CTRa alone, while 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 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.
Introduction

The definition of 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) (Hay et al., 2006; Poyner et al., 2002; Udawela et al., 2004). RAMPs are a family of 3 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 behaviour, 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 (AM), 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 also for the resultant phenotype of RAMP/CLR complexes (Fitzsimmons et al., 2003; Fraser et al., 1999; Kuwashako et al., 2001; Kuwashako et al., 2003). However, work with the CTR has revealed additional effects on phenotype that are dependent upon cellular background where co-expression of RAMP2 and CTRa (the predominantly human receptor splice variant) in CHO-P but not COS-7 cells led to 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 has
an additional 16 amino acids in intracellular loop 1 (Moore et al., 1995) in both CHO-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 while the N-terminus was the key domain for binding specificity of peptides, the transmembrane (TM) domain and/or C-terminus were important for the level of induced amylin binding and possibly for 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, USA). Oligonucleotide primers were synthesised by GeneWorks (Adelaide, SA, Australia). N-succinimidyl-3-(4-hydroxy-\(^{125}\)I-iodophenyl) propionate (Bolton-Hunter reagent; 200Ci/mmol) was supplied by Amersham Pharmacia Biotech (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 previously described (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 PCR technique (Barik and Galinski, 1991; Sarkar and Sommer, 1990). The N-terminal/transmembrane domain junction for the chimeras was the common aa sequence Asp-Pro-Pro at position 113-115 of RAMP1 and 140-142 of RAMP2, and the TM/C-terminal domain junction was the common aa sequence Leu-Val-Val at position 136-138 of RAMP1 and 163-165 of RAMP2 (internal primers 5’-ctgctggtgacggcacttgtagtatggagg-3’ and 5’-ccccctctctcatctctgtggctggcag-3’). The outer primers were designed to incorporate a HindIII site upstream of, and an XbaI site downstream of, the RAMP sequence (5’-catcaagttgccaccatgctgggccccctg-3’ and 5’-gcacctagatcactcactacatcgcctcagtg-3’ for
RAMP1 and 5'-catcaagcttgccaccatggcctcgctc-3' and 5'-gcaatctagatatctaggcctgggcctcactgtc-3' for RAMP2). PCR 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 Figure 1.

**Cell culture and transfections**

COS-7 cells were routinely maintained in 175 cm² flasks at 37°C in a humidified atmosphere with 5% CO₂; 95% air, in complete DMEM supplemented with 5% heat inactivated FBS, 100 units/ml penicillin-G and 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. 24 well plates were transfected with 100 ng receptor and 150 ng RAMP with 1 µl lipid, 75 cm² flasks with 4 µg receptor and 6 µg RAMP with 20 µl lipid and 25 cm² flasks with 1 µg receptor and 1.5 µg RAMP with 8 µl lipid.

**Receptor binding**

To determine specific binding, 48 h post transfection in 24-well plates, COS-7 cells were incubated with ~100 pM ¹²⁵I-rAmy in binding buffer (DMEM containing 0.1% BSA) in the absence (total binding) or presence (non-specific binding) of 1x10⁻⁶ M rAmy. After incubating at 37°C for 1 h, cells were washed with 1x phosphate buffered saline (PBS) and solubilised 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 cm² flasks and grown for 48 h, then harvested and resuspended in binding buffer (DMEM containing 1% BSA). Cells were added to 96 well plates (100,000 cells/well) with ∼70 pM ¹²⁵I-rAmy and competing unlabelled peptides. After incubating for 1h at 37°C, cells were harvested onto GF/C plates (coated with 0.5% polyvinylpyrolidone and 0.1% Tween20) using a Tomtec harvester. Plates were dried overnight and, after the addition of Micorscint 0’ (PerkinElmer Life Sciences; Boston, MA, USA), counted on a Topcount counter (PerkinElmer Life Sciences). Experiments were performed with triplicate repeats.

cAMP assays
Intracellular cAMP levels were determined using the AlphaScreen cAMP kit (PerkinElmer Life Sciences). Cells transfected in 25 cm² flasks were grown for 48 h, then serum starved overnight. Cells were then harvested, counted and incubated in stimulation buffer (phenol red free media, 0.1% BSA, 1 mM IBMX) for 20-30 min. Cells were added to wells containing agonists at 5,000 cells/well in 384-well plates. Following stimulation for 30 min at 37°C, lysis buffer (5 mM HEPES, 0.3% Tween20, 0.1% BSA) was added to all wells, with subsequent steps performed as previously described (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 presented as mean ± standard error of means. Curve fitting was done using Prism 4 (Graphpad Software Inc, San Diego, CA, USA). Comparison of pIC₅₀ or pEC₅₀ 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 $\rho IC_{50}$ values) or the CTa alone phenotype (for $\rho EC_{50}$ 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 towards induction of Amy binding, chimeras that individually exchange these two domains between RAMP1 and 2 were tested for induction of $^{125}$I-rAmy binding (Figure 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, while the reverse chimera, RAMP2/1, induced a high level of Amy binding, equivalent to that induced by RAMP1 (Figure 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 (Figure 2). Therefore, $^{125}$I-Amy binding levels, at least in the context of these chimeras, were principally influenced by the TMD of RAMPs.
Phenotype of AMY receptors induced by RAMP domain swap chimeras

The pharmacological profile induced by WT and chimeric RAMPs was further explored in peptide competition for $^{125}$I-Amy binding to COS-7 cells co-expressing 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 hCT while at the RAMP2/CTRa the order of potency was reversed (Figure 3 A, 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, where the affinity for hCGRP was higher than hCT (Figure 3 A, C; Table 1). In contrast, the RAMP2/1TMD induced phenotype was essentially equivalent to that induced by RAMP2 (Figure 3 B, D; Table 1). $^{125}$I-Amy binding to CTRa co-expressed 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 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 (Figure 4 A; Table 2). When RAMP1 was co-expressed the potency of hCGRP and rAmy increased, while RAMP2 co-expression
induced only a weak increase in rAmy potency (Figure 4 A versus B, C; Table 2). The phenotype induced by chimera RAMP1/2TMD was similar to that of RAMP1 (Figure 4 D; 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 co-transfected cells, however neither led to a change in hCGRP potency (Figure 4 E, F; Table 2). Interestingly, the RAMP1/2COOH chimera also had an ~10 fold decrease in potency for hCT (Figure 4 E; Table 2). In contrast the RAMP2/1COOH chimera induced phenotype that was essentially equivalent to that of RAMP1 (Figure 4 G versus B, C; Table 2), implying that the C-terminus of RAMPs affects signalling.
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-CGRP$\alpha$ was shown to cross-link to RAMP1 as well as CLR, indicating close proximity of RAMP1 and CLR at the cell membrane (McLatchie et al., 1998; Stangl et al., 1991). Second, Fraser et al. (1999) showed that the chimera RAMP1/2, which contains only the N-terminus of RAMP1, co-expressed with CLR in HEK29T cells, revealed a CGRP receptor similar to that seen with RAMP1, while the reverse chimera (RAMP2/1) revealed an adrenomedullin receptor similar to that induced by RAMP2 when co-expressed with CLR, suggesting that the N-terminal domain was sufficient to engender specificity of peptide interaction. Interestingly, the RAMP1/2 chimera gave a significantly higher level of $^{125}$I-adrenomedullin binding than wild-type RAMP1 (Fraser et al., 1999), suggesting 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), 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, while 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 is 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 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 behaviour. 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 likely to be an important contributor to the stability of RAMP/CTR complexes. Two recent papers have examined the role of RAMP1 domains in formation of the CGRP receptor (Fitzsimmons et al., 2003; Steiner et al., 2002). 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 HEK293 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 (PDGF) receptor, or the RAMP N-terminus alone, Fitzsimmons et al. (2003) showed that, while 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 destabilisation 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 (Udawela, Sexton unpublished data), as has been suggested by 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 signalling 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 signalling. The previous lack of investigation is in part due to the greater difficulty in interpretation of signalling data that is caused by the background CTR alone phenotype that is present in co-transfection experiments due to 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 co-transfection with CTRA resulted in an increase in hCGRP and rAMY potency compared to CTRA alone, while RAMP2 co-transfection 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 signalling profiles equivalent to that induced by RAMP1, while the potency of peptides for those constructs with the RAMP2 C-terminus (RAMP2/1TMD and RAMP1/2COOH) were similar to that seen with RAMP2. The minor exception to this was the RAMP1/2COOH construct where, in addition to increased rAmy potency,
there was a decrease in hCT potency. This latter behaviour 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 where a weaker increase in potency was observed where the RAMP2 C-terminus was present. This data is indicative of a critical role for the short C-terminal domain in the signalling 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 where deletion of 8 or 9 amino acids of the RAMP1 C-terminus had little effect on receptor signalling (Fitzsimmons et al., 2003; Steiner et al., 2002), possibly due to the importance of receptor component protein in signalling 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 stabilisation of RAMP-receptor complexes and the C-terminal domain in signalling profile of receptors. The data also highlight apparent differences between the behaviour of CTR/RAMP and CLR/RAMP complexes that may be important in targeting receptor complexes for drug development.
References


Footnotes

This work was supported by grants from the National Health and Medical Research Council of Australia (#299810) and the Ian Potter Neuropeptide Laboratory. PMS is a Principal Research Fellow of the NHMRC. AC is a Senior Research Fellow of the NHMRC.
Figure legends

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

Figure 2: Specific $^{125}I$-rAmy binding to COS-7 cells co-expressing CTRa and WT RAMPs or RAMP domain swap chimeras, expressed as percentage of RAMP1 induced binding. Experiments were carried out in 24-well plates using 100ng/well receptor and 150ng/well RAMPs. Total binding ranged from 2500 – 5000 cpm and non-specific binding ranged from 1000 – 2000 cpm for RAMP1 co-transfected 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).

Figure 3: Competition for $^{125}I$-Amy binding to COS-7 cells co-transfected with hCTRa and (A) RAMP1, (B) RAMP2, (C) RAMP1/2COOH or (D) RAMP2/1TMD 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.

Figure 4: Induction of cAMP accumulation in COS-7 cells co-transfected with hCTRa and (A) empty vector, (B) RAMP1, (C) RAMP2, (D) RAMP1/2TMD, (E) RAMP2/1TMD, (F) RAMP1/2COOH or (G) RAMP2/1COOH by hCGRPα (●).
rAmy (▲), hCT (▼) or sCT (■). Data are mean ± S.E.M. of four or more separate experiments, normalised to the maximal sCT response. $pEC_{50}$ values are given in Table 2.
Table 1: $pIC_{50}$ values for peptides in competition for $^{125}$I-rAmy binding to COS-7 cells co-transfected with hCTRa and WT or chimeric RAMPs. Data are mean ± S.E.M. ($n$≥4).

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<th>rAmy</th>
<th>*hCT</th>
<th>sCT</th>
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<td>CTRa + RAMP1</td>
<td>7.36 ± 0.15</td>
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<td>8.20 ± 0.18</td>
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<td>8.63 ± 0.18</td>
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*Significantly different between constructs (F-test; $P<0.05$)

**Significantly different from RAMP1, (unpaired t test; $P<0.05$)
Table 2:  

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<th>*rAmy</th>
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* Significantly different between constructs (F-test; *P<0.05)

**Significantly different from vector control (unpaired *t*-test; *P<0.05)
Figure 1
Figure 2
Figure 3
Figure 4