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The effects of C-terminal truncation of receptor activity modifying proteins on the induction of amylin receptor phenotype from human CTb receptors

Madhara Udawela [b,1], George Christopoulos [a,b], Maria Morfis [a,b], Nanda Tilakaratne [a], Arthur Christopoulos [a], Patrick M. Sexton [a,b,*]

[a] Drug Discovery Biology Laboratory, Department of Pharmacology, Monash University, Clayton, 3800, Victoria, Australia
[b] Howard Florey Institute, The University of Melbourne, Parkville, 3010, Victoria, Australia

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Abstract

Receptor activity modifying proteins (RAMPs) interact with calcitonin receptors to produce novel amylin receptor phenotypes. We have recently demonstrated that the short intracellular C-terminus of RAMPs plays a key role in the function of amylin receptors derived from the CTa calcitonin receptor through the use of chimeric RAMPs and RAMPs that are truncated at the C-terminus [15, Udawela M, Christopoulos G, Morfis M, Christopoulos A, Ye S, Tilakaratne N, Sexton PM. A critical role for the short intracellular C terminus in receptor activity modifying protein function. Mol Pharmacol 2006;70:1750–60., 18, Udawela M, Christopoulos G, Tilakaratne N, Christopoulos A, Albiston A, Sexton PM. Distinct receptor activity-modifying protein domains differentially modulate interaction with calcitonin receptors. Mol Pharmacol 2006;69:1984–89.]. The calcitonin receptor in humans is expressed as two major alternatively spliced isoforms termed CTa and CTb. Relatively little is known about how alternate splicing of the receptor affects the interaction between calcitonin receptors and RAMPs. We have examined the effect of RAMP truncation, through use of mutant constructs that delete the last 8 amino acids of each of the 3 known human RAMPs, and characterised these for interaction with CTb receptors through co-expression in COS-7 cells. As seen with the CTa receptor isoform, RAMP truncation caused a marked loss in induction of AMYb receptor phenotypes as characterised by ^{125}I-rat amylin radioligand binding assays and cAMP accumulation assays; the latter as a marker of receptor signalling. The effect was most pronounced for RAMP1 and RAMP2 deletion mutants, but attenuated responses were also observed with co-expressed RAMP3 deletion mutants. These data support a direct role for the RAMP C-terminus in the interaction of RAMP/calcitonin receptor complexes with intracellular accessory proteins involved in signalling and/or receptor trafficking.

Keywords: Calcitonin receptors; Receptor activity modifying proteins; Amylin receptor phenotypes

1. Introduction

Receptor activity modifying proteins (RAMPs) constitute a family of 3 single transmembrane proteins identified as prerequisite partners for the calcitonin receptor-like receptor (CLR) trafficking to the cell surface and expression of functional calcitonin gene-related peptide (CGRP) receptors ($CGRP_1$ receptor, CLR/RAMP1), or adrenomedullin receptors (AM_1, CLR/RAMP2; AM_2, CLR/RAMP3) [1–3]. Subsequent work revealed that RAMPs could also interact with calcitonin (CT) receptors to form different amylin receptor phenotypes (AMY_1, CTR/RAMP1; AMY_2, CTR/RAMP2; AMY_3, CTR/RAMP3) [3–6], each with their own distinct agonist and antagonist pharmacology [3,7,8]. Unlike CLR, which requires heterodimerization with RAMP to express a functional receptor at the cell surface [1,9], the CT receptor is well expressed at the cell surface in the absence of RAMPs and demonstrates the well characterized CT receptor phenotype with high affinity for mammalian CTs, such as human CT, and low affinity for the related peptides, amylin, CGRP and adrenomedullin [3,6].

Abbreviations: Δ; deletion mutant; AM; adrenomedullin; AMY; amylin receptor phenotype; CGRP; calcitonin gene-related peptide; CLR; calcitonin receptor-like receptor; CT; calcitonin; CTb receptor; calcitonin receptor b isoform; GPCR; G protein-coupled receptor; h; human; HA; haemagglutinin epitope tag; hCT; human calcitonin; PDZ; Post synaptic density-95/Discs-large/ZO-1 homology; RAMP; receptor activity modifying protein; rAmy; rat amylin; sCT; salmon calcitonin; TMD; transmembrane domain; WT; wild-type.

* Corresponding author. Tel.: +61 3 9905 1146.
E-mail address: patrick.sexton@med.monash.edu.au (P.M. Sexton).
[1] Current address: Baker Heart Research Institute, 75 Commercial Road, Prahran, Victoria, 3004, Australia.

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In man, the CT receptor has 2 major isoforms that arise from alternate RNA splicing, resulting in the absence (CTa) or presence (CTb) of a 16 amino acid insert in the predicted intracellular domain 1 [10–12]. These receptor isoforms differ in their capacity to signal via different pathways with a complete loss of Gq-mediated mobilization of intracellular calcium and an attenuation of cAMP signaling, downstream of Gs activation from the CTb receptor [11–13], although the extent of the effect on cAMP signaling varies across different cellular backgrounds [11–13]. In addition to the effect on signalling, the CTb isoform also exhibits a decreased level of receptor internalization [12], however, binding affinity and specificity for calcitonin peptides is not greatly affected [11–13]. Both CTa and CTb receptors can interact with RAMPs to form AMY receptors [14]; AMY receptors derived from the different CTR splice variants are identified according to the splice variant involved (eg. AMY_{1a}, CTa/RAMP1 or AMY_{1b}, CTb/RAMP1) [6]. However, there is a cell-background dependent difference in the formation of functional AMY receptors from the two CTR isoforms, with strong induction of ^{125}I-Amy binding seen for all 3 RAMPs with the CTb receptor in COS-7 cells, whereas only RAMPs 1 and 3 induce strong binding with the CTa receptor in this background [4,5,14,15]. In contrast, both receptor isoforms induce similar levels of ^{125}I-Amy binding for each RAMP in CHO-P cells [14], leading to speculation that the capacity of different CTR/RAMP complexes to interact with proteins such as G proteins may be altered [3].

RAMPs contain a short intracellular C-terminal tail of about 10 amino acids. The role of this domain is still unclear, but may be involved in differential cellular trafficking of AM_2 and AM_3 adrenomedullin receptors via interaction of the PDZ domain of RAMP3 with accessory proteins such as NHERF1 and NSF [16,17]. Recent data from chimeras between RAMP1 and RAMP2 have also implicated a role for the RAMP C-terminus in the signalling from RAMP/CTR heterodimers, with CGRP-induced accumulation of cAMP being strongly influenced by the C-terminal sequence in the chimeras [18]. These data suggest that the RAMP C-terminus could play a role in the coupling of receptor complexes to G proteins. Additional support for a role of the C-terminus in coupling of AMY receptors to G proteins came from analysis of RAMP mutants that lacked the last 8 amino acids, co-expressed with the CTa receptor, [15]. In that study, there was a marked loss of ^{125}I-Amy binding for deletion mutants of RAMP1 and RAMP2, and to a lesser extent RAMP3, that could be recovered, at least in part, by over-expression of Gαs. There was also a corresponding loss of signalling by this receptor via Gαs-mediated formation of cAMP. The loss of functional AMY receptors was also paralleled by a decrease in the capacity of the RAMP to be translocated to the cell surface by the CTa receptor, leading to speculation that coupling of the RAMP/CTR complex with G proteins may be important in stabilising the interaction [15]. The marked effect of RAMP C-terminal deletion on CTR-based receptors was in contrast to the relative lack of effect seen for equivalent CLR-based receptors, where only minimal loss of function was observed [15,19–21], although additional effects were seen in trafficking of these receptors in a cell-background dependent manner [15,21].

Given the profound effects seen on the CTa isoform of the receptor and the known differences in behaviour of the CTa and CTb receptors, both expressed alone and together with RAMPs, this study set out to examine whether the dependence of C-terminus of RAMP for induction of functional AMY receptors seen for the CTa receptor was also seen for the CTb splice variant.

2. 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 synthesized by GeneWorks (Adelaide, SA, Australia). Rabbit anti-c-Myc antibody was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). ^{125}I-labeled goat anti-mouse IgG was obtained from PerkinElmer (Boston, MA, USA). *N*-succinimidyl-3-(4-hydroxy-^{125}I-iodophenyl) propionate (Bolton–Hunter reagent; 2000 Ci/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 (HPLC) as previously described [22].

2.1. cDNA constructs

Expression clones of wild-type hRAMPs were provided by Dr S. M. Foord [9]. c-Myc-RAMP1 was provided by Dr K. Kuwasako [23]; this construct has equivalent distribution and behaviour to the wild-type RAMP1 [15,23]. Double HA epitope tagged human CTRb (HA-CTRb) was prepared as described previously [24]. This receptor is the Leu447 polymorphic variant of the receptor [12]. RAMP truncation mutants were prepared by QuickChange site-directed mutagensis (Stratagene, La Jolla, CA, USA), as previously described [15]. The resultant constructs are displayed schematically in Fig. 1.

2.2. Cell culture and transfections

COS-7 cells were routinely maintained in 175 cm^2 flasks at 37 °C in a humidified atmosphere with 5% CO_2: 95% air, in complete DMEM supplemented with 5% heat inactivated FBS, 100 units/ml penicillin-G and 100 μg/ml streptomycin and 50 μg/ml fungizone. These cells do not express functionally significant levels of either RAMPs or CTR [15]. Transfections were carried out in serum and antibiotic free DMEM using lipofectamine (Invitrogen) or Metafectene (Scientifix; Cheltenham, VIC, Australia), when cells were ~70% confluent. Cells grown in 24-well plates or 4-well chamber slides were transfected with 100 ng of expression vector containing receptor cDNA and 150 ng of expression vector containing RAMP cDNA mixed with 1 μl lipid; 75 cm^2 flasks with 4 μg of expression vector containing receptor cDNA and 6 μg of expression vector containing RAMP cDNA mixed with 20 μl lipid, and 25 cm^2 flasks with 1 μg receptor DNA and 1.5 μg RAMP DNA mixed with 8 μl lipid.