

Multiple Amylin Receptors Arise from Receptor Activity-Modifying Protein Interaction with the Calcitonin Receptor Gene Product

GEORGE CHRISTOPOULOS, KATIE J. PERRY, MARIA MORFIS, NANDA TILAKARATNE, YONGYI GAO, NEIL J. FRASER, MARTIN J. MAIN, STEVEN M. FOORD, and PATRICK M. SEXTON

Molecular Pharmacology Laboratory, Department of Pharmacology, The University of Melbourne, Victoria, Australia (G.C., K.J.P., M.M., N.T., Y.G., P.M.S.); and Receptor Systems Unit, Glaxo Wellcome Medicines Research Centre, Stevenage, Hertfordshire United Kingdom (N.J.F., M.J.M., S.M.F.)

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ABSTRACT

Receptor activity-modifying proteins (RAMPs) are single-transmembrane proteins that transport the calcitonin receptor-like receptor (CRLR) to the cell surface. RAMP 1-transported CRLR is a calcitonin gene-related peptide (CGRP) receptor. RAMP 2- or RAMP 3-transported CRLR is an adrenomedullin receptor. The role of RAMPs beyond their interaction with CRLR, a class II G protein-coupled receptor, is unclear. In this study, we have examined the role of RAMPs in generating amylin receptor phenotypes from the calcitonin (CT) receptor gene product. Cotransfection of RAMP 1 or RAMP 3 with the human CT receptor lacking the 16-amino acid insert in intracellular domain 1 (hCTR₁₁) into COS-7 cells induced specific ¹²⁵I-labeled rat amylin binding. RAMP 2 or vector cotransfection did not cause significant increases in specific amylin binding. Competition-binding characterization of the RAMP-induced amylin receptors revealed two distinct phenotypes. The RAMP 1-derived amylin receptor demonstrated the highest affinity for salmon CT (IC₅₀, 3.01 ± 1.44 × 10⁻¹⁰ M), a high to moderate affinity for rat amylin (IC₅₀, 7.86 ± 4.49 × 10⁻⁹ M) and human CGRP_α (IC₅₀,

2.09 ± 1.63 × 10⁻⁸ M), and a low affinity for human CT (IC₅₀, 4.47 ± 0.78 × 10⁻⁷ M). In contrast, whereas affinities for amylin and the CTs were similar for the RAMP 3-derived receptor, the efficacy of human CGRP_α was markedly reduced (IC₅₀, 1.12 ± 0.45 × 10⁻⁷ M; *P* < .05 versus RAMP 1). Functional cyclic AMP responses in COS-7 cells cotransfected with individual RAMPs and hCTR₁₁ were reflective of the phenotypes seen in competition for amylin binding. Confocal microscopic localization of c-myc-tagged RAMP 1 indicated that, when transfected alone, RAMP 1 almost exclusively was located intracellularly. Cotransfection with calcitonin receptor (CTR)₁₁ induced cell surface expression of RAMP 1. The results of experiments cross-linking ¹²⁵I-labeled amylin to RAMP 1/hCTR-transfected cells with bis succinimidyl suberate were suggestive of a cell-surface association of RAMP 1 and the receptors. Our data suggest that in the CT family of receptors, and potentially in other class II G protein-coupled receptors, the cellular phenotype is likely to be dynamic in regard to the level and combination of both the receptor and the RAMP proteins.

Amylin is a 37-amino acid pancreatic hormone that shares amino acid homology with the calcitonin gene-related peptide (CGRP), calcitonin (CT), and the adrenomedullin family of peptides. It has the highest identity (~45%) with the CGRPs, an ~22% identity with the 38 C-terminal amino acids of adrenomedullin, and an 18 and 33% identity (with a gapped alignment) with rat/human and avian/teleost CTs, respectively. The physiology of these peptides has been reviewed in detail (Muff et al., 1995; Wimalawansa, 1997). Circulating

levels of amylin are raised in response to meal ingestion, and the peptide acts to potently inhibit gastric emptying, postprandial glucagon secretion, and food intake. Amylin also opposes the metabolic actions of insulin in skeletal muscle (Sexton and Perry, 1996; Young, 1997). Transgenic mice lacking the amylin gene show abnormal weight gain, an observation that also suggests an important metabolic role for amylin (Devine and Young, 1998; Gebre-Medhin et al., 1998). An independent gene encoding the amylin receptor has not been identified.

McLatchie et al. (1998) recently identified and cloned a family of accessory proteins termed receptor activity-modify-

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ABBREVIATIONS: CGRP, calcitonin gene-related peptide; CT, calcitonin; RAMP, receptor activity-modifying protein; CRLR, calcitonin receptor-like receptor; α -TSH cells, α -thyroid-stimulating hormone thyrotroph cells; BS³, bisuccinimidyl suberate; cAMP, cyclic AMP; CHO, Chinese hamster ovary; CTR, calcitonin receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; hCTR₁₁, human CT receptor lacking the 16 amino acid insert in intracellular domain 1; HEK, human embryonic kidney; rCTR₁₁, rat insert negative CT receptor isoform equivalent to rat C1a CT receptor.

ing proteins (RAMPs), which was comprised of three members designated RAMP 1, RAMP 2, and RAMP 3. These single-transmembrane domain proteins induced trafficking of the calcitonin receptor-like receptor (CRLR) to the cell surface, where it exhibited either a CGRP receptor phenotype (RAMP 1) or adrenomedullin receptor phenotypes (RAMP 2 or RAMP 3). RAMPs therefore provided a novel mechanism for engendering novel receptor phenotypes. Amylin shows more sequence homology to CGRP than adrenomedullin, but it does not activate or bind to combinations of CRLR and RAMPs (McLatchie et al., 1998). Amylin has even less sequence identity with the CTs, but there is evidence that links amylin receptors with those for CT. The receptors tend to be colocalized (Sexton and Perry, 1996), and both receptors are recognized by antibodies raised against the hypervariable C terminus of the CT receptor (Perry et al., 1997). Furthermore, transfection of human CT receptors into human embryonic kidney (HEK)-293 cells, but not into *Ti ni* insect cells, induces low levels of amylin receptors in addition to high levels of CT receptors (Chen et al., 1997). Moreover, transfection of the porcine CT receptor into different cellular backgrounds gives rise to different receptor phenotypes with transfection into Chinese hamster ovary (CHO)-K1 cells, yielding a receptor similar to the rat C1a CT receptor (Christmanson et al., 1994), whereas transfection into COS-7 or HEK-293 cells yields a receptor with moderate to high affinity for amylin and poor responsiveness to human CT (Christmanson et al., 1994; Sexton et al., 1994a).

These observations prompted an investigation of whether RAMP coexpression may also underlie the expression of amylin receptors from the CT receptor gene product. Our data indicate that at least two independent amylin receptor phenotypes may be engendered by specific RAMP interaction.

Experimental Procedures

Materials. Salmon CT, human CT, human adrenomedullin, human CGRP α , and rat amylin were obtained from Bachem (Torrance, CA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), HEPES, G418, and Lipofectamine were obtained from GIBCO-BRL Life Technologies (Grand Island, NY). BSA was obtained from Commonwealth Serum Laboratories (Parkville, Australia), anti-c-myc antibody was obtained from Invitrogen (Carlsbad, CA), and Alexa 488-conjugated goat anti-mouse sera and TOTO-3 were obtained from Molecular Probes (Eugene, OR). Isobutylmethylxanthine was obtained from Sigma Chemical Co. (St. Louis, MO), tissue culture plates and flasks were obtained from Nunc (Roskilde, Denmark), and anti-cyclic AMP (cAMP) antibody was a gift from Dr. Philip Marley (Department of Pharmacology, University of Melbourne, Melbourne, Australia). Endo F was obtained from Boehringer Mannheim (Mannheim, Germany), and bissuccinimidyl suberate (BS³) was obtained from Pierce Chemical Co. (Rockford, IL). Na¹²⁵I and ¹²⁵I-labeled rat amylin (specific activity, 2000 Ci/mmol) were obtained from Amersham (Buckinghamshire, UK). ¹²⁵I-labeled salmon CT (specific activity, ~700 Ci/mmol) was prepared as described previously (Nicholson et al., 1986). All other chemicals were of reagent grade or better.

Cell Culture and cDNA Transfection. Green monkey kidney COS-7 cells were maintained in 175-cm² flasks at 37°C in a humidified atmosphere with 95% O₂:5% CO₂, in complete DMEM supplemented with 10% FBS, 80 mg/l gentamycin, 1 mg/l minocycline, and 15 mM HEPES. Human embryonic kidney (HEK)-293 cells stably expressing the rat C1a CT receptor [clones F12 (~60,000 receptors/cell) and D11 (~600,000 receptors/cell); Houssami et al., 1994] were

maintained in antibiotic- and HEPES-supplemented DMEM containing 5% FBS and 200 μ g/ml G418. CHO-K1 cells (a gift from Dr. Steve Rees, GlaxoWellcome Medicines Research Center, Stevenage, UK) were maintained in DMEM/Ham's F12 media (50:50) supplemented with 10% FBS, 2 mM glutamine, and 0.5 mg/ml hygromycin B.

Coexpression of CT Receptor and RAMP cDNA. Cells in 24- or 6-well plates were grown to 70 to 80% confluency and transfected with 0.1 μ g (unless otherwise specified) of plasmid DNA encoding the most commonly expressed CT receptor isoforms from human [hCTR₁₁; a gift from Dr. Emma Moore, Zymogenetics Inc., Seattle, WA (Kuestner et al., 1994)] or rat (C1a; Albrandt et al., 1993; Sexton et al., 1993) and RAMP cDNA (McLatchie et al., 1998) per 2 cm² by using Lipofectamine, according to the manufacturer's instructions. The CT receptor isoforms from rat and human are equivalent in their exon splicing and have been denoted hCTR₁₁ and rCTR₁₁ for this study. In some experiments, increasing concentrations of RAMP DNA were transfected. Radioligand binding and cAMP assays were performed 48 h after transfection.

Radioligand Binding. Receptor-expressing cells in 24-well plates, at 90 to 100% confluency, were incubated in binding buffer [DMEM containing 0.1% (w/v) BSA] with ~80 pM ¹²⁵I-labeled salmon CT or ~70 pM ¹²⁵I-labeled rat amylin (Sexton et al., 1993), in the absence (total binding) or presence of increasing concentrations of unlabeled ligands. Nonspecific binding was defined as binding in the presence of 1 μ M homologous unlabeled peptide. After incubation for 60 min at 37°C, cells were washed with ice-cold PBS (140 mM NaCl, 2 mM KCl, 1 mM KH₂PO₄, and 8 mM Na₂HPO₄, pH 7.3) and solubilized with 0.5 M NaOH. Competition binding curves were analyzed with the Equilibrium Binding Data Analysis/Ligand software package (Biosoft, Cambridge, UK).

cAMP Assay. Transfected cells in 24-well plates, at 90 to 100% confluency, were preincubated in cyclase buffer [DMEM containing 0.1% (w/v) BSA and 1 mM isobutylmethylxanthine] for 20 min at 37°C. Cells subsequently were incubated for 25 min in the absence (basal) or presence of increasing concentrations of ligand. After incubation, cells were washed with ice-cold PBS, and cAMP was extracted with 0.5 ml of absolute ethanol. cAMP levels were assayed by radioimmunoassay as described previously (Sexton et al., 1994a).

Covalent Cross-Linking Analysis. Transfected cells in six-well plates were incubated for 60 min in binding buffer with an ~4 nM concentration of the specified radioligand in the absence (total binding), or presence of 1 μ M homologous unlabeled peptide (nonspecific binding). After incubation, cells were washed with PBS and cross-linked on ice for 35 min with 1 mM BS³. Cells were collected and solubilized in sample buffer [50 mM Tris HCl (pH 6.8) containing 2% (w/v) SDS, 0.1% bromophenol blue, 10% (v/v) glycerol, and 100 mM dithiothreitol] and centrifuged at 12,000g for 30 min at 4°C, and the supernatants were analyzed by 10% (w/v) SDS-polyacrylamide gel electrophoresis (Quiza et al., 1997). Gels were stained with Coomassie blue R-250, destained, dried, and exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA). Deglycosylation was performed as described previously (Quiza et al., 1997). The *M_r* of labeled bands was determined from a standard curve generated from the electrophoretic mobility of molecular weight markers that were co-electrophoresed with the samples.

Confocal Microscopic Localization of c-myc-Tagged RAMP

1. RAMP 1 epitope tagged with the c-myc epitope at the N terminus (McLatchie et al., 1998) was transfected transiently into COS-7 cells seeded onto 22-mm glass coverslips in six-well plates, either alone or together with the I1⁻ isoform of the rat or human CT receptor. Then, 48 h after transfection, cells were fixed with 3.2% paraformaldehyde for 30 min at 22°C, the reaction was stopped with 150 mM glycine in PBS, and the cells were washed three times for 5 min in either PBS or PBS containing 0.3% Triton X-100. All subsequent treatments and washes were performed in either PBS for cell surface labeling or in PBS-Triton to allow intracellular identification of epitope-tagged protein. Cells were preblocked with 10% lamb serum for 30 min at

22°C, washed once with PBS or PBS-Triton, and then incubated with anti-c-myc antisera at a dilution of 1:500 for 1 h at 22°C. After incubation with primary antisera, cells were washed once and then incubated in the dark with Alexa 488-conjugated goat anti-mouse sera (1:100) for 1 h at 22°C. Cells were further incubated in the dark with PBS-Triton containing TOTO-3 (1:1000) and RNase (1:250) for 1 h, washed twice with PBS-Triton for 5 min, dipped in distilled water, and mounted onto glass slides with fluorescent mounting media. Cells were visualized with a Bio-Rad confocal microscope (Bio-Rad Laboratories, Hercules, CA).

Results

Cotransfection of hCTR₁₁ with increasing concentrations of RAMP 1 or RAMP 3 into COS-7 cells induced specific and high-affinity ¹²⁵I-labeled amylin binding (Fig. 1, a and e). RAMP 2 had no significant effect (Fig. 1, a and e). Similar results were seen with the rCTR₁₁ receptor isoform (Fig. 1b). In contrast, in HEK-293 cells stably expressing the rCTR₁₁, only RAMP 1 induced alteration in the level of specific ¹²⁵I-labeled amylin binding (Fig. 1c). No significant change was seen in the level of ¹²⁵I-labeled salmon CT binding for any of the CT receptors studied (Fig. 1, d and f).

Transfection of increasing levels of RAMP into CHO-K1 cells, which endogenously express CT receptors, demonstrated induction of ¹²⁵I-labeled amylin binding with RAMPs 1 and 3 but not with RAMP 2 or a vector control (Fig. 2). The expression of RAMP alone into COS-7 cells did not enable binding of either ¹²⁵I-labeled amylin or ¹²⁵I-labeled salmon CT (not shown).

The amylin receptors generated by the coexpression of the hCTR₁₁ isoform with RAMP 1 and with RAMP 3 were analyzed in competition binding studies. K_d values for amylin and salmon CT were 4.48×10^{-9} M and 9.43×10^{-10} M, respectively, for CTR/RAMP 1 and 5.38×10^{-9} M (amylin) and 5.8×10^{-9} M (salmon CT) for CTR/RAMP 3. The hCTR₁₁/RAMP 1 combination generated an amylin receptor equivalent to that identified previously in α -thyroid-stimulating hormone thyrotroph cells (α -TSH cells; Perry et al., 1997). It had the highest affinity for salmon CT, a high to moderate affinity for rat amylin and human CGRP α , and a low affinity for human CT (Fig. 3a; Table 1). Human adrenomedullin had little interaction with this receptor, being at least 10-fold less potent than human CT (not shown). In

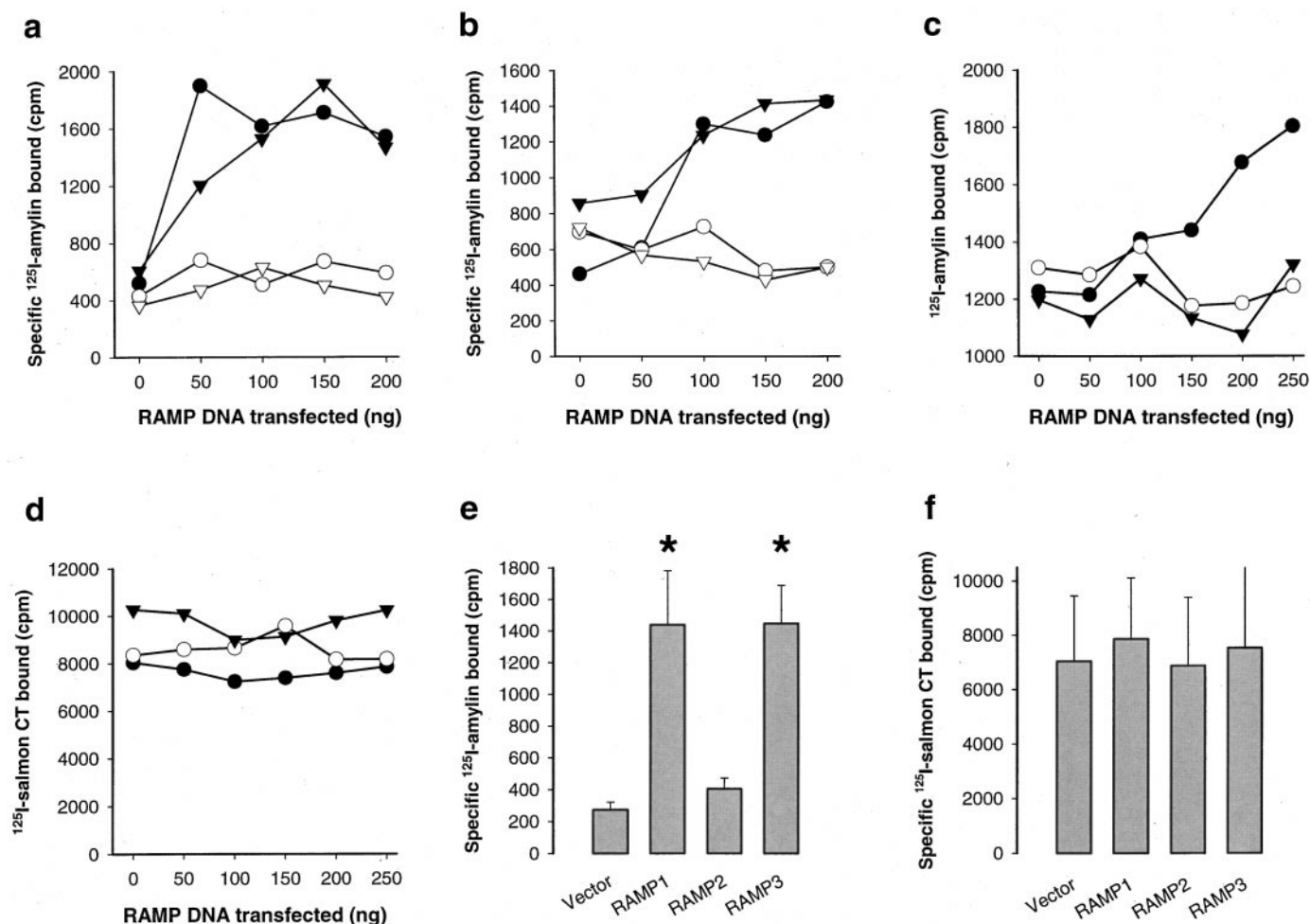


Fig. 1. Effect of cotransfection of RAMP 1, RAMP 2, RAMP 3, or vector DNA on the expression of ¹²⁵I-labeled amylin (a, b, c, e) or ¹²⁵I-labeled salmon CT (d, f) binding in COS-7 cells transiently transfected with 100 ng of CT receptor (a, b, e, f) or HEK-293 cells stably expressing the rCTR₁₁ (c, d). a, ¹²⁵I-labeled amylin binding in cells transfected with 100 ng of hCTR₁₁. b, ¹²⁵I-labeled amylin binding in cells transfected with 100 ng of rCTR₁₁. c, ¹²⁵I-labeled amylin binding in cells stably expressing the rat CTR₁₁. d, ¹²⁵I-labeled salmon CT binding in cells stably expressing the rCTR₁₁. Representative experiments ($n \geq 3$) with triplicate repeats. e and f, pooled data for specific ¹²⁵I-labeled amylin binding (e) or ¹²⁵I-labeled salmon CT binding (f) after cotransfection with 100 ng of RAMP and 100 ng of hCTR₁₁. *, $P < .05$, ANOVA, multiple comparisons versus control (Tukey's Test; $n = 7$). ●, RAMP 1. ○, RAMP 2. ▼, RAMP 3. ▽, vector.

contrast, the binding of ^{125}I -labeled amylin to the hCTR₁₁/RAMP 3 combination was competed for by salmon CT, amylin, and human CT in a manner similar to that seen with hCTR₁₁/RAMP 1, but human CGRP α was ~30-fold less effective (Fig. 3b; Table 1). As for the RAMP 1-induced phenotype, human adrenomedullin had the lowest affinity for this receptor and essentially did not compete for binding except at micromolar concentrations (not shown). Little change was seen in the level and specificity of ^{125}I -labeled salmon CT binding to COS-7 cells after cotransfection with any of the RAMPs (Table 2). Similar results were seen with the rCTR₁₁ isoform (not shown).

The functional cAMP responses in COS-7 cells cotransfected with individual RAMPs and hCTR₁₁ were consistent with the pharmacology of the amylin binding they induced. Cells cotransfected with receptor and vector control showed responses typical of a CT receptor (Kuestner et al., 1994; Albrandt et al., 1995; Gorn et al., 1995), with salmon and human CT displaying similar efficacy and amylin and CGRP only weakly stimulating cAMP accumulation. RAMP 1 and RAMP 3 increased amylin potency (Fig. 4a), whereas only RAMP 1 increased CGRP potency (Fig. 4b). RAMP cotransfection caused a decrease in the efficacy of human CT (Fig. 4c), whereas the efficacy of salmon CT essentially was unaltered by RAMP treatment (Fig. 4d). Consistent with its limited effect on specific amylin binding, RAMP 2 had little effect on peptide specificity and potency (Fig. 4).

BS³ cross-linking of ^{125}I -labeled amylin to RAMP 1/hCTR₁₁-transfected cells revealed a broad receptor-binding protein with a M_r of ~80,000, whereas cells transfected with receptor plus vector control exhibited essentially no specific amylin binding (Fig. 5). ^{125}I -labeled salmon CT labeled a band with a M_r of ~80,000 in both RAMP 1- and vector

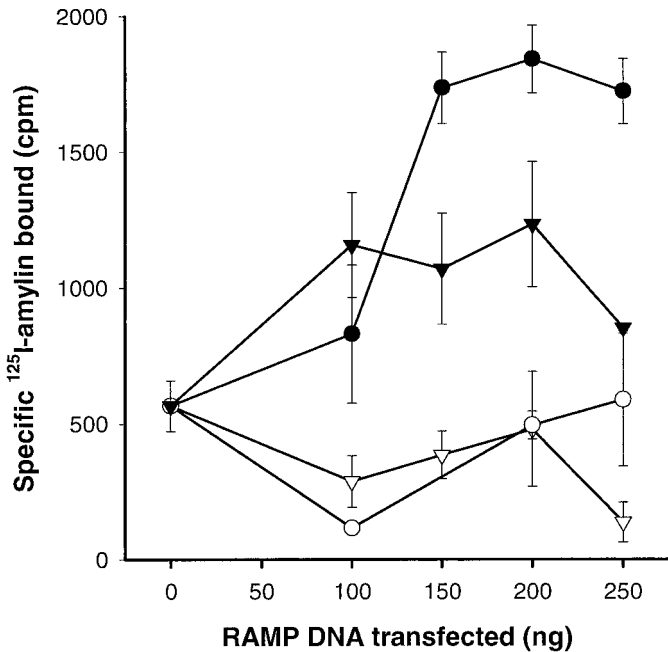


Fig. 2. Effect of transfection of RAMP 1 (●), RAMP 2 (○), RAMP 3 (▼), or vector (▽) DNA on the expression of ^{125}I -labeled amylin binding in CHO-K1 cells that endogenously express a native CT receptor. RAMP 1 and RAMP 3, but not RAMP 2 or vector DNA, increased specific ^{125}I -labeled amylin binding. Data are from a representative experiment with triplicate repeats ($n =$ three separate experiments).

control-transfected cells. Endo F deglycosylation reduced the size of the ^{125}I -labeled salmon CT-binding protein to a M_r of ~54,000, consistent with the predicted size of the core recep-

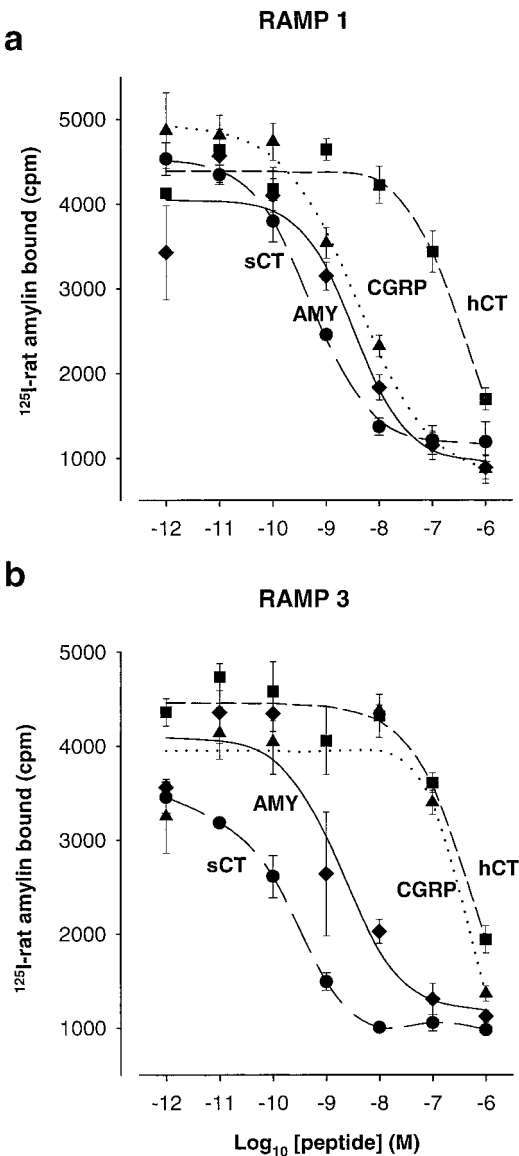


Fig. 3. RAMP 1 and RAMP 3 generate two distinct amylin receptors. a, competition of ^{125}I -labeled rat amylin binding to COS-7 cells cotransfected with 100 ng of hCTR₁₁ and 100 ng of RAMP 1 DNA. b, competition of ^{125}I -labeled amylin binding to cells cotransfected with hCTR₁₁ and RAMP 3 DNA. Salmon CT (sCT, ●), rat amylin (AMY, ◆), human CGRP α (CGRP, ▲), human CT (hCT, ■). Data are from a single representative experiment with triplicate repeats ($n = 3$). IC₅₀ values for pooled data are shown in Table 1.

TABLE 1
IC₅₀ values (nM) for peptides in competition for ^{125}I -labeled rat amylin binding to COS-7 cells cotransfected with 100 ng of hCTR₁₁ and 100 ng of RAMP ($n = 3$)

| | RAMP 1 | RAMP 3 | α -TSH Cells* |
|---------------------|-------------------|-------------------|----------------------|
| Competing peptide | | | |
| Rat amylin | 7.86 \pm 4.49 | 6.35 \pm 1.70 | 10.5 \pm 0.32 |
| Salmon CT | 0.309 \pm 0.144 | 0.206 \pm 0.004 | 0.37 \pm 0.10 |
| Human CT | 447 \pm 78 | 862 \pm 619 | N.D. |
| Human CGRP α | 4.68 \pm 3.04 | 151 \pm 48** | 5.95 \pm 1.70 |

* From Perry et al., 1997.
** $P < .05$ RAMP 1 versus RAMP 3 ($n = 3$).

tor protein (Quiza et al., 1997). In contrast, deglycosylation of the ^{125}I -labeled amylin-binding protein with Endo F generated two distinct bands, a lower band with a M_r of $\sim 54,000$ and an upper band with a M_r of $\sim 68,000$ (Fig. 5). Similar results were seen in HEK-293 cells stably transfected with rCTR₁₁- receptor homolog and transfected with RAMP 1 (not shown).

Confocal microscopic localization of RAMP 1 incorporating a c-myc epitope tag in the N terminus revealed that, when transfected alone, little of the protein was expressed on the

cell surface (Fig. 6, a and b). However, when cotransfected with rCTR₁₁-, significant cell-surface expression of the protein was observed (Fig. 6c).

Discussion

The discovery of RAMPs and the elucidation of their role in the trafficking of CRLR and its expressed cell-surface phenotype provided a novel potential mechanism for the diversification and the regulation of receptor function. However, the

TABLE 2

IC₅₀ values (M) for peptides in competition for ^{125}I -labeled salmon CT binding to COS-7 cells cotransfected with 100 ng of hCTR₁₁- and 100 ng of RAMP ($n = 3$)

| Competing peptide | Receptor Alone | RAMP1 | RAMP2 | RAMP3 |
|---------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Rat amylin | $5.37 \pm 2.84 \times 10^{-7}$ | $1.72 \pm 0.73 \times 10^{-7}$ | $8.78 \pm 1.96 \times 10^{-8}$ | $1.96 \pm 0.95 \times 10^{-7}$ |
| Salmon CT | $9.33 \pm 6.08 \times 10^{-10}$ | $2.24 \pm 0.30 \times 10^{-10}$ | $1.34 \pm 0.46 \times 10^{-10}$ | $3.17 \pm 0.35 \times 10^{-10}$ |
| Human CT | $1.55 \pm 0.23 \times 10^{-8}$ | $2.95 \pm 0.52 \times 10^{-8}$ | $8.27 \pm 1.57 \times 10^{-9}$ | $1.97 \pm 1.06 \times 10^{-8}$ |
| Human CGRP α | $>10^{-6}$ | $>10^{-6}$ | $>10^{-6}$ | 10^{-6} |

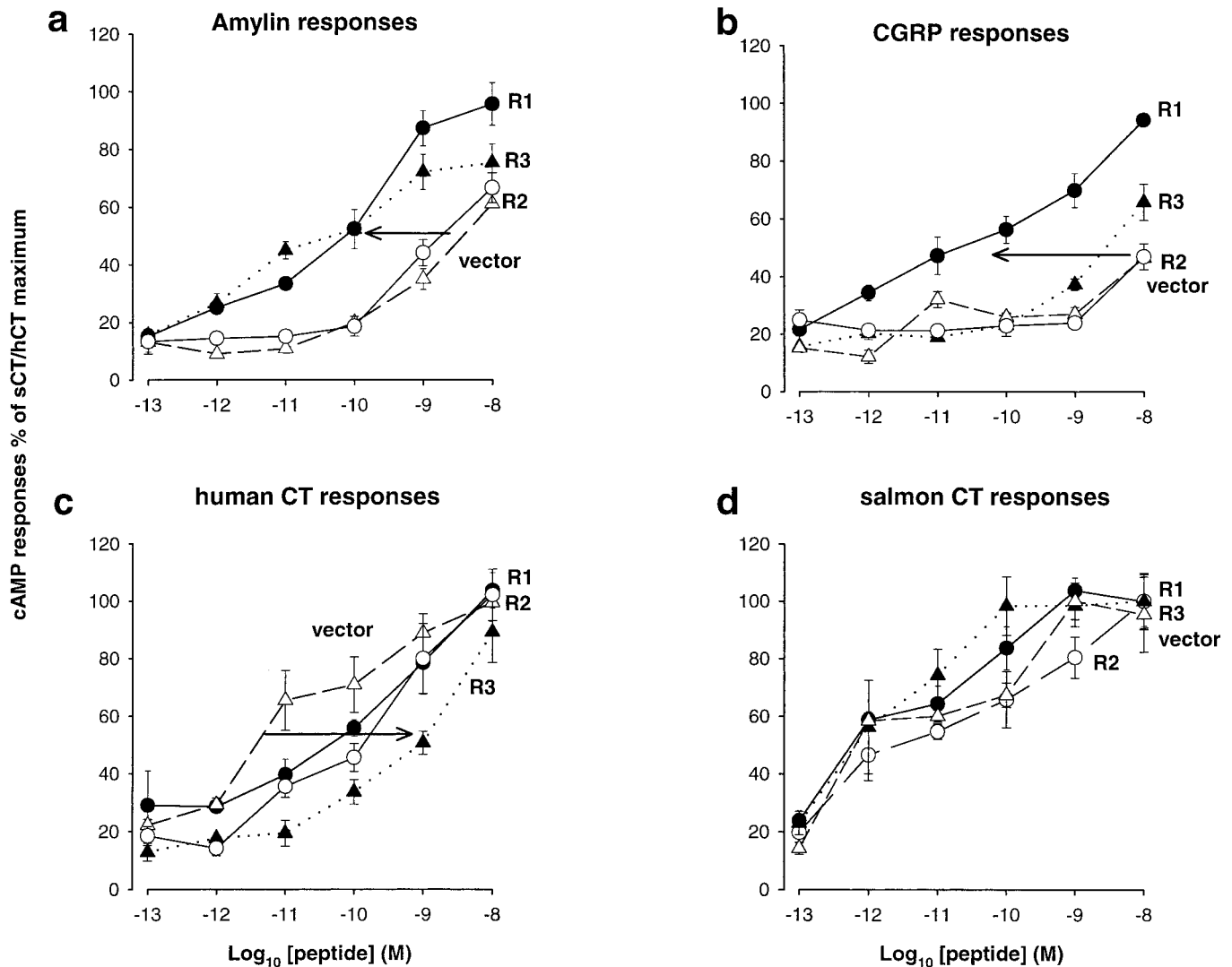


Fig. 4. Effect of RAMPs on ligand-induced cAMP responses in COS-7 cells cotransfected with 100 ng of hCTR₁₁- and 100 ng of vector control (Δ), RAMP 1 (\bullet), RAMP 2 (\circ), or RAMP 3 (\blacktriangle) DNA. a, amylin responses; b, CGRP responses; c, human CT responses; d, salmon CT responses. Data are from a single representative experiment ($n = 3$). Arrows indicate the direction of potency shift for each of the peptides in response to RAMP cotransfection. Basal cAMP levels were ~ 350 , 600, 720, and 400 pmol/ml/ 10^6 cells for vector-, RAMP 1-, RAMP 2-, and RAMP 3-cotransfected cells, respectively. Maximal responses were ~ 2150 , 2700, 3800, and 2200 pmol/ml/ 10^6 cells for vector-, RAMP 1-, RAMP 2-, and RAMP 3-cotransfected cells, respectively.

role of RAMPs beyond their interaction with CRLR is unclear. CRLR shares ~55% amino acid sequence identity with the CT receptor and is almost 80% identical in the transmembrane regions. This homology suggested that the CT receptor protein might also be a target for RAMP interaction. In this study, we demonstrate that RAMPs do indeed interact with the CT receptor gene product to induce novel receptor phenotypes. RAMP 1 cotransfection with CT receptors generated an amylin receptor equivalent to that identified in mouse α -TSH cells (Hanna et al., 1995; Perry et al., 1997). The profile of peptide interaction was also similar to amylin receptors characterized in the nucleus accumbens (Beaumont et al., 1993), the kidney (Wookey et al., 1996), and skeletal muscle (Pittner et al., 1996). Although the affinity of peptides interacting with the nucleus accumbens appears higher (Beaumont et al., 1993), this is likely attributable to, at least in part, the difference in assay format, with live cells being used in the current study and membranes being used for the measurement of nucleus accumbens binding. Indeed, analysis of binding competition in brain slices also yields lower affinity for competing peptides (Sexton et al., 1994b).

Comparison of the RAMP 1- and RAMP 3-induced receptor phenotypes indicates that there are different forms of amylin receptor with differential sensitivity to CGRP, and there is evidence for this in tissue preparations. Differential sensitivity of amylin binding to competition by CGRP within rat brain nuclei has been suggested by the results of autoradiographic studies (van Rossum et al., 1994). The disparity in affinity is modest, ~10-fold at most, but it is consistent with the difference between RAMP 1- and RAMP 3-induced receptor profiles. Amylin binding to regions such as the dorsomedial and arcuate hypothalamic nuclei (low CGRP affinity) is consistent predominantly with the RAMP 3-induced phenotype. Amylin receptors in the nucleus accumbens core and the amygdala (high CGRP affinity) resemble the RAMP 1-induced phenotype. Elsewhere, the affinity of CGRP is intermediate, which may imply varying levels of mixed-re-

ceptor phenotypes. Both RAMP 1 and RAMP 3 are expressed significantly in brain (McLatchie et al., 1998). For cells in which an amylin receptor phenotype is induced, it is unclear why the relative potency of ligands in competition for 125 I-labeled salmon CT binding is not significantly altered. However, it is likely that cells cotransfected with CT receptor and

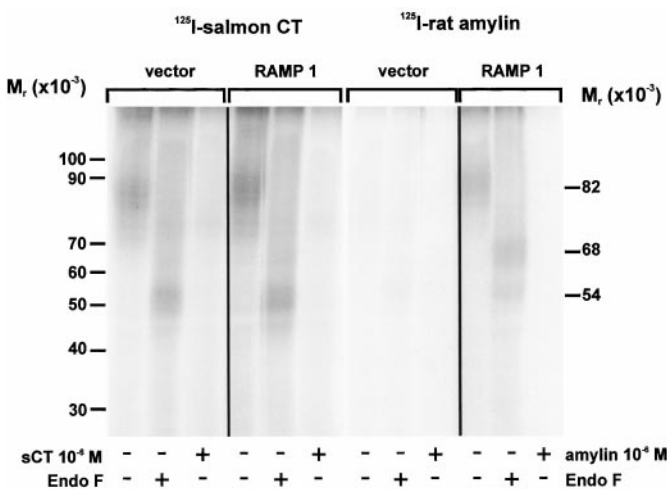


Fig. 5. Transfection of RAMP 1 with hCTR₁₁ into COS-7 cells enables BS³ to specifically cross-link 125 I-labeled rat amylin to a broad glycosylated protein with a M_r of ~80,000. Binding of 125 I-labeled sCT essentially was unaltered by cotransfection of RAMP 1. However, after Endo F deglycosylation of cross-linked RAMP 1 transfected cells, two bands with a M_r of ~68,000 and ~54,000 were seen with 125 I-labeled amylin. In cells transfected with hCTR₁₁ alone, only the lower M_r band was seen after deglycosylation of 125 I-labeled salmon CT cross-linked receptor. The data are from an individual representative experiment ($n = 3$).

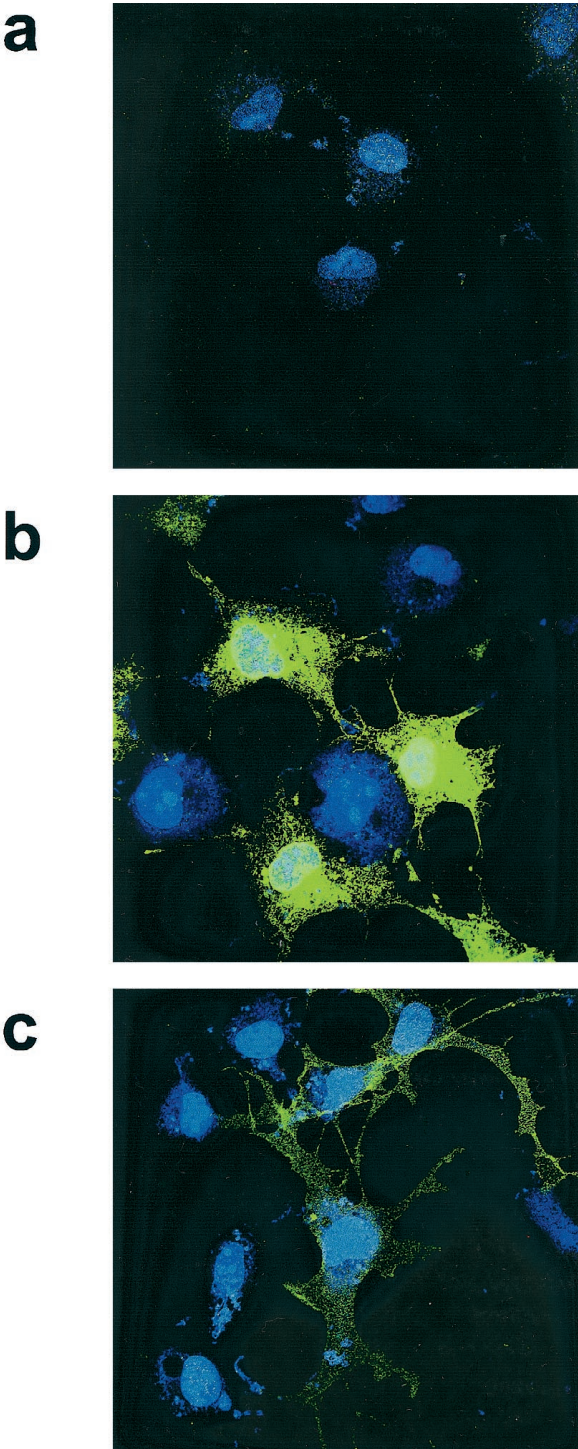


Fig. 6. Confocal microscopic localization of c-myc RAMP 1 transfected alone (a, b) or with the rCTR₁₁ (c). a and c, cell surface labeling in nonpermeabilized cells. b, labeling in cells permeabilized with 0.3% Triton X-100. The data are from an individual representative experiment ($n = 6$). The data indicate the strong induction of cell-surface expression of RAMP 1 with receptor coexpression.

RAMP 1 or RAMP 3 express mixed amylin-CT receptor phenotypes. Furthermore, we have speculated that specificity of peptides in competition ^{125}I -labeled salmon CT binding is more reflective of affinity for inactive state receptor (Housami et al., 1995).

These data show that in cells expressing CTR_{11-} , RAMP expression determines the extent to which they respond to the CT family of peptides. For the majority of experiments, the expression of CTR_{11-} , through cotransfection with RAMP, occurs together with RAMP. However, we also have demonstrated that RAMP expression gives rise to novel amylin receptors in CHO-K1 cells endogenously expressing CT receptors and in cells stably expressing the rCTR_{11-} . In the latter cell line, unlike COS-7 cells, only RAMP 1 was capable of inducing amylin-receptor binding, which suggests that cellular background, including native RAMP levels and, potentially, other components such as G protein levels, plays a significant role in the derived receptor phenotype.

As observed previously for CGRP and adrenomedullin (McLatchie et al., 1998), the expression of RAMP alone did not enable binding of either amylin or salmon CT, indicating that RAMPs are not receptors by themselves. Confocal microscopic analysis of RAMP 1 distribution indicated that little cell-surface expression of the protein occurred when transfected alone, although significant intracellular protein expression was observed. Cotransfection of CT receptor with RAMP 1 induced the appearance of the RAMP at the cell surface, as has been observed previously with cotransfection of CRLR and RAMP 1 (McLatchie et al., 1998). However, unlike CRLR, which does not traffic to the cell surface in the absence of RAMP, CT receptor alone is strongly expressed at the cell surface, yielding a classical CT receptor phenotype. Thus, for the CT receptor gene product, RAMP appears to be acting principally as a phenotypic modulator and not as a trafficking protein. However, the possibility that RAMP may affect the processing and trafficking of newly formed CT receptor protein in the expression of novel receptor phenotypes cannot be excluded.

Although inconclusive, the results of deglycosylation studies with ^{125}I -labeled amylin cross-linked to cell surface-expressed amylin receptor were suggestive of an association between the "CT receptor" protein and a protein the size of which was equivalent to RAMP 1, with the appearance of a band with a M_r of $\sim 64,000$, in addition to the core protein band with a M_r of $\sim 54,000$. Although it is possible that the higher-molecular-weight band reflects partial deglycosylation of the receptor, we believe that this is unlikely, because the vast majority of the salmon CT-binding protein runs as core protein under equivalent conditions. Thus, the data may indicate a close cell surface association of RAMP 1 (BS^3 cross-links primary amino groups within $\sim 20 \text{ \AA}$) and the CT receptor gene product in the expression of the amylin receptor phenotypes. Although differences in the pattern of glycosylation between the major amylin- and salmon CT-binding proteins occur in α -TSH cells (Perry et al., 1997), no apparent differences were seen in the current study, suggesting that alteration in the level of glycosylation is not required for the expression of amylin receptor phenotype. For the α -TSH amylin receptor, the additional carbohydrate occurred at a site susceptible to deglycosylation under nondenaturing conditions (Perry et al., 1997). We have shown previously that

glycosylation at a similar site occurs in CT receptors expressed in CT-receptor-naïve cells in the absence of the overt expression of an amylin receptor phenotype (Quiza et al., 1997), and that this carbohydrate may be removed without affecting ligand binding. It remains possible, however, that small changes in either the degree or the site of glycosylation, beyond the resolution of the current experiments, do occur. At the CRLR, terminal modification of the carbohydrate is induced by RAMP 1 in generating the high affinity CGRP receptor (McLatchie et al., 1998), although it is unclear whether this modification contributes directly to CGRP binding. Nonetheless, the induction of a novel receptor phenotype by RAMP does not necessarily involve alterations to receptor glycosylation as RAMP 2- or RAMP 3-induced adrenomedullin-like receptors occur without carbohydrate modification (McLatchie et al., 1998).

We have demonstrated for the first time that receptors for amylin can be created by the coexpression of CT receptors and RAMPs. The novel amylin receptors have pharmacologies consistent with those observed in tissue preparations. RAMPs have now been shown to determine the pharmacology of both CT receptor and CRLR. These receptors are homologous, particularly in their transmembrane domains. The physiological significance of these regulatory mechanisms is uncertain, but they suggest a way in which cells could change their sensitivity to peptides in the CT/CGRP family and possibly other class II G protein-coupled receptors.

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Send reprint requests to: Dr. Patrick M. Sexton, Department of Pharmacology, University of Melbourne, Parkville 3052, Victoria, Australia. E-mail: p.sexton@pharmacology.unimelb.edu.au
