

Identification of N-Terminal Receptor Activity-Modifying Protein Residues Important for Calcitonin Gene-Related Peptide, Adrenomedullin, and Amylin Receptor Function^[S]

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ABSTRACT

Calcitonin-family receptors comprise calcitonin receptor-like receptor (CL) or calcitonin receptor and receptor activity-modifying protein (RAMP) pairings. Calcitonin gene-related peptide (CGRP) receptors are CL/RAMP1, whereas adrenomedullin (AM) receptors are CL/RAMP2 (AM₁ receptor) or CL/RAMP3 (AM₂ receptor). Amylin (Amy) receptors are RAMP hetero-oligomers with the calcitonin receptor (AMY₁, AMY₂, and AMY₃, respectively). How RAMPs change G protein-coupled receptor pharmacology is not fully understood. We exploited sequence differences between RAMP1 and RAMP3 to identify individual residues capable of altering receptor pharmacology. Alignment of human RAMPs revealed eight residues that are conserved in RAMP2 and RAMP3 but are different in RAMP1. We hypothesized that residues in RAMP2 and RAMP3, but not RAMP1, are responsible for making CL/RAMP2 and CL/RAMP3 AM receptors. Using site-directed mutagenesis, we introduced individual

RAMP3 residues into RAMP1 and vice versa in these eight positions. Mutant or wild-type RAMPs were transfected into Cos7 cells with CL or the insert-negative form of the calcitonin receptor [CT_(a)]. Agonist-stimulated cAMP production and cell-surface expression of constructs were measured. Position 74 in RAMP1 and RAMP3 was critical for determining AM potency and affinity, and Phe93 in RAMP1 was an important contributor to αCGRP potency at CGRP receptors. Mutant RAMP/CT_(a) receptor complexes displayed different phenotypes. It is noteworthy that RAMP1 S103N and W74E mutations led to enhanced rAmy potency, probably related to increased cell-surface expression of these complexes. This differs from the effect on CL-based receptors where expression was unchanged. Targeted substitution has emphasized the importance of position 74 in RAMP1/RAMP3 as a key determinant of AM pharmacology.

The calcitonin peptide family, in humans, comprises calcitonin, amylin (Amy), two forms of calcitonin gene-related peptide (CGRP; α and β) and adrenomedullin (AM). A second version of AM, a 47-amino acid peptide named intermedin (IMD) or AM2, has been identified more recently (Fig. 1A)

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(Roh et al., 2004; Takei et al., 2004). Calcitonin-peptide family receptors are composed of G protein-coupled receptors and receptor activity-modifying proteins (RAMPs) (Poyner et al., 2002). For example, AM receptors are heteromers of the calcitonin receptor-like receptor (CL) with RAMP2 (AM₁ receptors) or RAMP3 (AM₂ receptors). CGRP₁ receptors are formed from CL/RAMP1 complexes. In contrast, Amy receptors comprise the calcitonin receptor with individual RAMPs to generate AMY₁, AMY₂, and AMY₃ receptor subtypes.

Pharmacological profiles have been determined for most of these receptor complexes in heterologous expression systems (Christopoulos et al., 1999; Nagoshi et al., 2002; Hay et al., 2003, 2005; Kuwasako et al., 2003a; Bailey and Hay, 2006). Several interesting observations can be made from these

ABBREVIATIONS: AMY, amylin receptor phenotype; AM, adrenomedullin; BIBN4096BS, 1-piperidinecarboxamide, *N*-[2-[[5-amino-1-[4-(4-pyridinyl)-1-piperazinyl]carbonyl]pentyl]amino]-1-[(3,5-dibromo-4-hydroxyphenyl)methyl]-2-oxoethyl]-4-(1,4-dihydro-2-oxo-3(2*H*)-quinazolinyl); CGRP, calcitonin gene-related peptide; CL, calcitonin receptor-like receptor; CT_(a), insert negative calcitonin receptor isoform; HA, hemagglutinin epitope tag; IMD, intermedin; IMDS, intermedin-short; RAMP, receptor activity modifying protein; WT, wild type; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ALPHA, amplified luminescent proximity homogenous assay.

studies. For example, RAMP1- and RAMP3-based receptors expressed with CL (i.e., CGRP₁ and AM₂ receptors) show reverse pharmacology (CGRP > AM or AM > CGRP, respectively) (Hay et al., 2006a). On the other hand, intermedin, in particular the short form (40 amino acids, IMDS), did not discriminate between the three RAMP/CL complexes (Roh et al., 2004; Hay et al., 2005). Such observations raise interesting questions about the features of the RAMPs, receptors, and the peptides that are important for their respective pharmacology.

The mechanism of peptide interactions has been studied in the most detail at CL/RAMP-based receptors. For these receptors, the long N termini of the proteins seemingly act in concert to provide binding sites for CGRP and AM (Hilalret et al., 2001). Studies involving RAMP1/2 chimeras or RAMP1 truncation/deletion mutants indicate that the RAMP N terminus is the major determinant of receptor pharmacology (Fraser et al., 1999; Zumpfe et al., 2000; Steiner et al., 2002; Fitzsimmons et al., 2003; Udawela et al., 2006).

Deletion mutants or chimeras of RAMPs implicate human RAMP2 residues 86 to 92 in high-affinity AM binding; in human RAMP3, it is the homologous residues 59 to 65 (Kuwasako et al., 2001). In RAMP1, the equivalent residues (59–65) are also needed for high-affinity interactions with CGRP and AM (Kuwasako et al., 2003b). However, alanine scans of these regions in RAMP2 showed that no single amino acid substitution caused a significant perturbation of cAMP production. Data from chimeric RAMPs suggested that the main AM binding epitope in RAMP2 was located between residues 77 and 101, but several deletion mutants within this region gave poor cell-surface expression, precluding further analysis. In human RAMP1, residues 41 to 45, 59 to 65, 67 to 71, and 91 to 103 were needed for high-affinity CGRP interactions. Deletion of residues 78 to 80 and 88 to 90 allowed cAMP production by CGRP but not by AM (Kuwasako et al., 2003b).

Although the RAMP N terminus is clearly a key determinant of pharmacological selectivity, it has not been confirmed whether the RAMP provides the appropriate conformation to the receptor for peptide binding or whether it provides direct points of contact. However, there has been some success in

understanding the binding mechanism of the CGRP antagonist, BIBN4096BS, in which Trp74 in RAMP1 plays a key role in the very high-affinity binding and selectivity of this compound for human CGRP₁ receptors (Mallee et al., 2002; Hay et al., 2003, 2006b; Bailey and Hay, 2006; Salvatore et al., 2006). RAMP3-based AM₂ receptors are relatively insensitive to BIBN4096BS antagonism but can be made more sensitive by mutating the native glutamic acid at position 74 to tryptophan (E74W) (Hay et al., 2006b). It is interesting that the E74W RAMP3 mutant also has another deviation from wild-type (WT) in that AM potency is reduced at the E74W AM₂ receptor (Hay et al., 2006b). Moreover, CGRP interactions with this receptor were not changed, suggesting that this substitution disrupts a natural interaction site for AM but not CGRP.

Sequence alignment of human RAMPs reveals that RAMP2 also has glutamic acid at the equivalent position (Glu101) to Glu74 in RAMP3 (Fig. 1B). This suggests that glutamic acid is an important amino acid in AM receptors at this position and perhaps helps to confer the greater affinity for AM that these receptors have over the CGRP₁ receptor. It is interesting that there are another seven positions in the N termini of human RAMPs that fit this pattern (i.e., are the same in RAMP2 and RAMP3 but different in RAMP1) (Fig. 1B). Therefore, in this study, we hypothesized that these residues may play a role in defining RAMP1/CL as a CGRP receptor and RAMP2/CL or RAMP3/CL as an AM receptor.

Materials and Methods

DNA Constructs. Human CL with an N-terminal hemagglutinin (HA) epitope tag (HA-CL), human RAMP1 with an N-terminal myc tag (mycRAMP1), and human RAMPs 2 and 3 were kindly provided by Steven M. Foord (GlaxoSmithKline, Stevenage, UK) and have been described previously (McLatchie et al., 1998). HA-tagged human HA-CT_(a) was the leucine polymorphic variant (Pham et al., 2004). We used mycRAMP1 because its behavior has been shown to be equivalent to that of WT RAMP1 (McLatchie et al., 1998). However, epitope-tagged RAMP3 constructs have significant differences from WT RAMP3 (M. Udawela, unpublished data), and therefore untagged RAMP3 was used in this study.

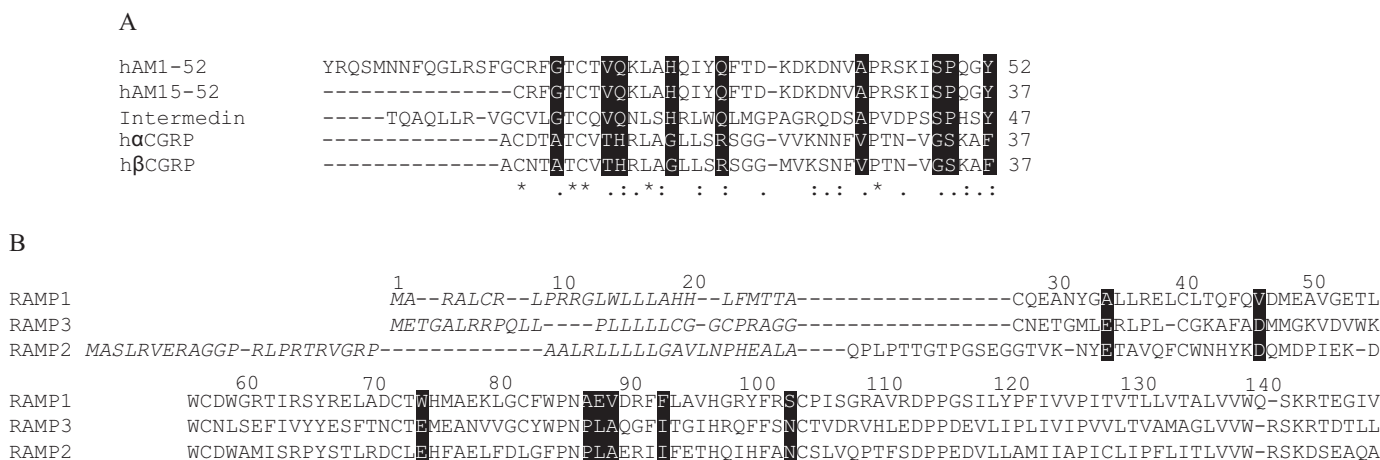


Fig. 1. A, amino acid sequence alignment of human AMs with CGRPs. Black-highlighted residues with white text are conserved in the AM peptides but are different in CGRPs. B, alignment of human RAMP amino acid sequences. Residues beyond the predicted signal sequences (shown in italics), which are conserved in the RAMP N termini between RAMPs 2 and 3 but are unique in RAMP1, are highlighted. The black bar represents the approximate position of the transmembrane region.

TABLE 1

Summary of cAMP data for human RAMP3 mutants containing individual RAMP1 residues expressed with HA-CL

E_{\max} values are cAMP percentage of forskolin. * $p < 0.05$, ** $p < 0.01$ versus WT by unpaired t test. Further data for α CGRP at E74W AM₂ receptors were published in Hay et al. (2006b).

Mutant	hAM				h α CGRP			
	pEC ₅₀ \pm S.E.M. (n)		E_{\max} \pm S.E.M. (n)		pEC ₅₀ \pm S.E.M. (n)		E_{\max} \pm S.E.M. (n)	
	WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant
E35A AM ₂	9.92 \pm 0.15 (3)	9.92 \pm 0.11 (3)	72.4 \pm 2.89 (3)	70.1 \pm 4.91 (3)	7.5 \pm 0.15 (4)	7.27 \pm 0.11 (4)	85.4 \pm 3.76 (4)	84.0 \pm 3.23 (4)
D46V AM ₂	9.92 \pm 0.15 (3)	9.91 \pm 0.11 (3)	72.4 \pm 2.89 (3)	66.4 \pm 4.81 (3)	7.50 \pm 0.15 (4)	7.40 \pm 0.08 (4)	83.5 \pm 2.00 (4)	84.6 \pm 0.48 (4)
E74W AM ₂	10.1 \pm 0.14 (5)	9.21 \pm 0.14** (5)	66.5 \pm 2.86 (5)	65.3 \pm 4.86 (5)	7.06 (1)	7.63 (1)	78.3 (1)	84.7 (1)
P87A AM ₂	9.92 \pm 0.13 (3)	10.2 \pm 0.09 (3)	70.3 \pm 5.69 (3)	75.8 \pm 4.87 (3)	7.44 \pm 0.13 (4)	7.41 \pm 0.03 (4)	84.9 \pm 3.64 (4)	84.1 \pm 2.51 (4)
L88E AM ₂	9.96 \pm 0.10 (4)	10.0 \pm 0.06 (4)	67.8 \pm 4.73 (4)	66.2 \pm 5.38 (4)	7.47 \pm 0.14 (4)	7.34 \pm 0.07 (4)	83.2 \pm 2.51 (4)	80.2 \pm 3.17 (4)
A89V AM ₂	9.71 \pm 0.15 (3)	10.3 \pm 0.13* (3)	69.3 \pm 10.2 (3)	71.2 \pm 8.79 (3)	7.39 \pm 0.15 (4)	7.70 \pm 0.03 (4)	83.3 \pm 2.49 (4)	78.1 \pm 2.04 (4)
I93F AM ₂	9.81 \pm 0.14 (4)	9.93 \pm 0.04 (4)	66.8 \pm 7.64 (4)	62.6 \pm 9.78 (4)	7.36 \pm 0.14 (4)	7.10 \pm 0.14 (4)	83.5 \pm 2.81 (4)	81.1 \pm 2.55 (4)
N103S AM ₂	9.99 \pm 0.18 (3)	10.1 \pm 0.09 (3)	73.6 \pm 3.79 (3)	68.9 \pm 6.45 (3)	7.32 \pm 0.16 (4)	7.27 \pm 0.05 (4)	81.4 \pm 3.53 (4)	79.5 \pm 1.89 (4)

TABLE 2

Summary of cAMP data for human RAMP3 mutants containing individual RAMP1 residues expressed with HA-CT_(a)

E_{\max} values are cAMP percentage of forskolin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus WT by unpaired t test. Values for HA-CT_(a) alone: for rAmy, pEC₅₀ \pm S.E.M.: 8.85 \pm 0.09 (n = 6), and E_{\max} \pm S.E.M.: 89.4 \pm 2.05 (n = 6); for h β CGRP, pEC₅₀ \pm S.E.M.: 8.25 \pm 0.04 (n = 8) and E_{\max} \pm S.E.M.: 80.9 \pm 4.64 (n = 8).

Mutant	rAmy				h β CGRP			
	pEC ₅₀ \pm S.E.M. (n)		E_{\max} \pm S.E.M. (n)		pEC ₅₀ \pm S.E.M. (n)		E_{\max} \pm S.E.M. (n)	
	WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant
E35A AMY _{3(a)}	10.3 \pm 0.16 (3)	9.85 \pm 0.25 (3)	83.6 \pm 1.63 (3)	77.2 \pm 5.02 (3)	9.36 \pm 0.14 (4)	9.16 \pm 0.28 (4)	89.3 \pm 5.77 (4)	74.5 \pm 6.57 (4)
D46V AMY _{3(a)}	10.3 \pm 0.16 (3)	10.3 \pm 0.22 (3)	85.8 \pm 3.38 (3)	73.7 \pm 6.75 (3)	9.36 \pm 0.14 (4)	9.37 \pm 0.23 (4)	89.3 \pm 5.77 (4)	79.5 \pm 5.51 (4)
E74W AMY _{3(a)}	10.7 \pm 0.08 (4)	9.87 \pm 0.09*** (4)	88.1 \pm 2.13 (4)	76.8 \pm 5.54 (4)	10.1 \pm 0.11 (3)	9.60 \pm 0.14* (3)	85.1 \pm 1.27 (3)	81.8 \pm 2.60 (3)
P87A AMY _{3(a)}	10.6 \pm 0.14 (4)	10.2 \pm 0.12 (4)	93.0 \pm 4.41 (4)	79.2 \pm 8.41 (4)	10.1 \pm 0.11 (3)	9.83 \pm 0.21 (3)	85.6 \pm 0.88 (3)	80.4 \pm 4.92 (3)
L88E AMY _{3(a)}	10.7 \pm 0.13 (3)	10.2 \pm 0.20 (3)	95.2 \pm 5.44 (3)	84.6 \pm 6.76 (3)	10.1 \pm 0.10 (3)	9.73 \pm 0.12 (3)	86.0 \pm 1.32 (3)	79.4 \pm 1.93* (3)
A89V AMY _{3(a)}	10.7 \pm 0.13 (3)	10.5 \pm 0.02 (3)	95.2 \pm 5.44 (3)	86.1 \pm 6.78 (3)	10.0 \pm 0.11 (3)	10.2 \pm 0.13 (3)	85.3 \pm 1.73 (3)	85.9 \pm 4.68 (3)
I93F AMY _{3(a)}	10.7 \pm 0.17 (4)	10.1 \pm 0.07* (4)	89.3 \pm 1.77 (4)	84.6 \pm 2.20 (4)	10.0 \pm 0.07 (4)	9.14 \pm 0.13*** (4)	84.9 \pm 2.02 (4)	75.5 \pm 3.38 (4)
N103S AMY _{3(a)}	10.8 \pm 0.15 (3)	9.69 \pm 0.08** (3)	87.2 \pm 1.16 (3)	80.2 \pm 5.23 (3)	10.1 \pm 0.07 (3)	9.06 \pm 0.02*** (3)	86.0 \pm 2.88 (3)	75.5 \pm 1.72* (3)

Site-Directed Mutagenesis. Forward and reverse oligonucleotide primers were designed with single base changes to incorporate amino acid point mutations in mycRAMP1, RAMP2, and RAMP3.

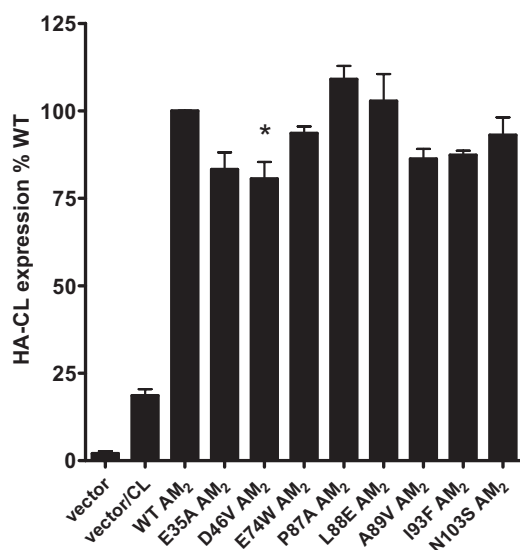


Fig. 2. ELISA data for RAMP3 mutants expressed with HA-CL (AM₂ receptors), measuring HA-CL expression at the cell surface. *, $p < 0.05$ versus WT by one-way ANOVA followed by Dunnett's test. Data are mean \pm S.E.M. of four independent experiments, each performed with eight replicates.

This method has been described previously (Hay et al., 2006b; Bailey and Hay, 2007). All primers were custom-synthesized by Invitrogen (Carlsbad, CA). Primer sequences are available on request.

Cell Culture and Transfection. Culture of Cos7 cells was performed essentially as described previously (Bailey and Hay, 2006). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% heat-inactivated fetal bovine serum and 5% v/v penicillin/streptomycin and kept in a 37°C humidified 95% air/5% CO₂ incubator. Cells were plated into 96-well plates (cAMP/ELISA) or 175-cm² flasks ([¹²⁵I]hAM₁₃₋₅₂ binding) 1 day before transfection. Cells were transiently transfected using polyethylenimine as described in detail in a previous publication (Bailey and Hay, 2006). Plasmid DNA was 250 ng in total per well of each 96-well plate or 160 μ g per flask and was incubated with polyethylenimine in 5% glucose for approximately 10 min before being added to complete growth medium. Cells were used for experimentation 36 to 48 h later. For [¹²⁵I]rAmy binding (selected mycRAMP1 mutants), cAMP and antibody binding experiments of the mutants E74D/E74Q/E74K AM₂ and E101W AM₁ and appropriate WT controls, Cos7 cell culture, and transfection were as described previously (Hay et al., 2005, 2006b).

cAMP Assay. Most cAMP assays were performed using a radio-receptor assay as described previously (Bailey and Hay, 2006). In brief, transfected cells were serum-deprived in DMEM containing 1 mM isobutyl methylxanthine and 0.1% bovine serum albumin for 30 min before the addition of agonists and incubation at 37°C for 15 min. cAMP was extracted with absolute ethanol and measured. In experiments using E74D/E74Q/E74K AM₂ and E101W AM₁ receptor mutants, cells were harvested approximately 40 h after transfection, and cAMP assays were performed as described previously (Hay et al.,

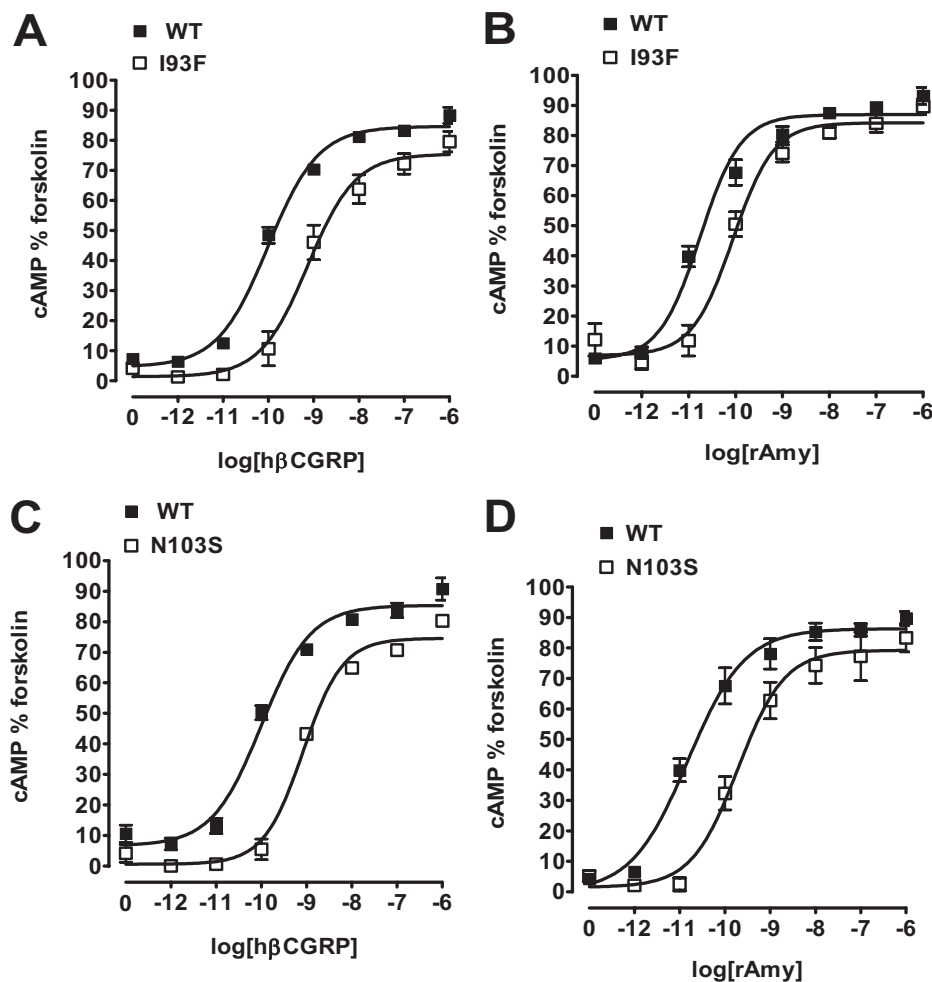


Fig. 3. cAMP data for RAMP3 mutants I93F and N103S expressed with HA-CT_(a). I93F (A and B) and N103S (C and D) with hβCGRP and rAmy, respectively. Data are mean \pm S.E.M. of three to four independent experiments, performed in triplicate.

2005, 2006b), where cAMP content was assayed by amplified luminescent proximity homogenous assay (ALPHA) screen.

Cell-Based ELISA and [¹²⁵I]IgG Binding. In most experiments, ELISA was used to measure the cell-surface expression of HA-CL or mycRAMP1, depending on the primary antibody used, to measure the expression of CGRP₁ and AM₂ or AMY_{1(a)} receptor complexes, respectively. This method is a modified form of that published by Versteeg et al. (2000). Our modifications have been published previously (Bailey and Hay, 2007). The myc antibody (Calbiochem, San Diego, CA) was used at a dilution of 1:250. Values were normalized to WT expression levels as 100%. For E74D/E74Q/E74K AM₂ and E101W AM₁ complexes, cell-surface expression was assessed by detecting [¹²⁵I]IgG (goat anti-mouse) bound to the N-terminal HA tag on CL (Hay et al., 2005, 2006b).

[¹²⁵I]rAmy Binding. [¹²⁵I]rAmy binding was performed to evaluate rAmy affinity of selected mutant receptor complexes. [¹²⁵I]rAmy binding was undertaken on whole cells in 48-well plates as described previously (Hay et al., 2005, 2006b). The only modification was that cells in the presence of 100,000 cpm of [¹²⁵I]rAmy in each well with or without unlabeled rAmy were incubated for 45 min at 37°C in a CO₂ incubator.

Membrane Preparation. Membranes of cells transfected with mutant RAMPs E74W/W74E with CL were harvested as described previously (Bailey and Hay, 2007). The only modification was that a 1:100 dilution of protease inhibitor cocktail (P8340; Sigma, St. Louis, MO) was added to the membrane harvest buffer. In brief, cells transfected and grown in two T-175 cm² flasks for each transfection mix were removed using a cell scraper and combined. The dislodged cells were homogenized and centrifuged. The pellet was resuspended and rehomogenized. A small aliquot was retained for protein content assay, and the remainder was frozen at -80°C.

[¹²⁵I]hAM₁₃₋₅₂ Binding. [¹²⁵I]hAM₁₃₋₅₂ binding was performed on the membrane preparations described above in duplicate in siliconized microcentrifuge tubes (Bio Plas Inc., San Rafael, CA). A dilution series of nonradiolabeled hAM was prepared in binding buffer (20 mM HEPES, 5 mM MgCl₂ · 6H₂O, 5 mM KCl, 10 mM NaCl, 1 mM EDTA-disodium, pH 7.4, and 0.1% bovine serum albumin), giving final concentrations ranging from 10⁻⁶ (nonspecific binding) to 10⁻¹³ M (mutant W74E) or 10⁻¹¹ M (mutant E74W). [¹²⁵I]hAM₁₃₋₅₂ was diluted with binding buffer to allow approximately 30,000 cpm in 50 μl (~9.1 pM). Membranes were thawed on ice and rehomogenized with a glass homogenizer if the membranes were not an even suspension. A 50-μl diluted radiolabel, 50-μl appropriate unlabeled competitor from the dilution series or buffer (total binding), and 76- to 100-μg membranes were added to each assay tube in this order. The mixture was vortexed briefly and incubated at 4°C for 30 min. The membranes were pelleted in a bench-top microcentrifuge at 4°C for 5 min. The supernatants were carefully removed, and the pellets were washed once with 500 μl of ice-cold binding buffer. Pellets were counted for γ radiation for 1 min.

Data Analysis and Statistical Procedures. Data were analyzed using GraphPad Prism (versions 4.02 or 5.01; GraphPad Software Inc., San Diego, CA). cAMP data generated from the radioreceptor assay were normalized to the response obtained to 50 μM forskolin that was present as a control on each plate. The quantity of cAMP generated from the ALPHA screen was calculated from the raw data using a cAMP standard curve. cAMP data from both assays were fitted to obtain pEC₅₀ and E_{max} values using a four-parameter logistic equation. For statistical analysis, pEC₅₀ and E_{max} values were compared between WT and mutant receptors using unpaired *t* tests. Expression values were compared using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. [¹²⁵I]hAM₁₃₋₅₂ binding data (in counts per minute) were first converted to femtomoles of radiolabel bound per milligram of membrane protein; specific binding was calculated for each data point that was then fitted to obtain pIC₅₀ values. The maximum specific binding (in femtomoles per milligram) for mutant versus WT receptors were compared by unpaired *t* test. [¹²⁵I]rAmy binding data (on whole cells) were expressed as specific binding, which was normalized to the

TABLE 3
Summary of cAMP data for human mycRAMP1 mutants containing individual RAMP3 residues expressed with HA-CL
E_{max} values are cAMP percentage of forskolin. * *p* < 0.05, ** *p* < 0.01 versus WT by unpaired *t* test.

Mutant	hAM				hαCGRP			
	pEC ₅₀ ± S.E.M. (n)		E _{max} ± S.E.M. (n)		pEC ₅₀ ± S.E.M. (n)		E _{max} ± S.E.M. (n)	
	WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant
A34E CGRP1	8.27 ± 0.19 (3)	8.21 ± 0.07 (3)	68.7 ± 5.84 (3)	71.6 ± 0.85 (3)	10.2 ± 0.07 (3)	10.6 ± 0.07* (3)	74.5 ± 0.27 (3)	80.3 ± 0.96** (3)
V46D CGRP1	8.50 ± 0.16 (3)	8.32 ± 0.10 (3)	77.0 ± 1.48 (3)	74.8 ± 2.78 (3)	10.2 ± 0.11 (3)	10.6 ± 0.13 (3)	78.7 ± 3.61 (3)	85.5 ± 4.13 (3)
W74E CGRP1	8.43 ± 0.15 (5)	9.26 ± 0.08** (5)	70.4 ± 2.65 (5)	74.9 ± 3.20 (5)	10.5 ± 0.20 (5)	10.6 ± 0.20 (5)	75.8 ± 3.88 (5)	77.4 ± 3.75 (5)
Δ87P CGRP1	8.50 ± 0.15 (3)	8.41 ± 0.09 (3)	74.7 ± 1.17 (3)	76.3 ± 2.85 (3)	10.2 ± 0.16 (3)	10.6 ± 0.25 (3)	80.3 ± 2.79 (3)	85.7 ± 5.13 (3)
E88L CGRP1	8.68 ± 0.16 (3)	8.48 ± 0.06 (3)	70.1 ± 5.29 (3)	72.1 ± 3.68 (3)	10.3 ± 0.14 (3)	10.3 ± 0.29 (3)	79.4 ± 2.31 (3)	75.6 ± 0.85 (3)
V89A CGRP1	7.81 ± 0.43 (3)	7.70 ± 0.15 (3)	72.4 ± 3.25 (3)	76.0 ± 3.02 (3)	9.86 ± 0.18 (3)	9.62 ± 0.19 (3)	73.1 ± 5.58 (3)	75.5 ± 1.93 (3)
F93I CGRP1	8.03 ± 0.25 (3)	7.60 ± 0.18 (3)	70.4 ± 2.08 (3)	73.1 ± 4.35 (3)	10.1 ± 0.15 (3)	9.40 ± 0.12* (3)	72.2 ± 4.79 (3)	66.9 ± 4.98 (3)
S103N CGRP1	8.23 ± 0.29 (3)	8.20 ± 0.16 (3)	69.8 ± 3.33 (3)	69.9 ± 2.20 (3)	10.1 ± 0.25 (3)	10.5 ± 0.16 (3)	79.3 ± 3.43 (3)	73.9 ± 3.78 (3)

maximum binding for each experiment. Data were fitted to obtain pIC_{50} , and maximum specific binding for multiple receptors was compared by one-way ANOVA followed by Dunnett's test. Significance was achieved at $p < 0.05$.

Drugs, Chemicals, and Other Materials. Human (h) α CGRP, h β CGRP, hIMD, and hAM were purchased from Bachem (Bubendorf, Switzerland). The 15 to 52 fragment of hAM (AM₁₅₋₅₂) was kindly provided by Professor David Coy (Tulane University Medical School, New Orleans, LA). Rat Amy (rAmy) was purchased from American Peptide (Sunnyvale, CA), Auspep (Parkville, VIC, Australia), or Bachem. Peptides were dissolved in water to make 1 mM stock solutions (taking into account peptide content) and stored as aliquots at -30°C in siliconized microcentrifuge tubes. Isobutyl methylxanthine, protein kinase A, and activated charcoal were from Sigma (St. Louis, MO). DMEM, fetal bovine serum, and HEPES were from Invitrogen. Forskolin was from Tocris (Ellisville, MO). Metafectine was purchased from Scientifix (Cheltenham, VIC, Australia). ALPHA screen cAMP kits were purchased from PerkinElmer (Waltham, MA). ^{125}I -Labeled goat anti-mouse IgG was obtained from PerkinElmer. Na- ^{125}I (100 mCi/ml) was supplied by ICN Biomedicals (Irvine, CA). *N*-Succinimidyl 3-(4-hydroxy-5- ^{125}I -iodophenyl) propionate (Bolton-Hunter reagent; 2000 Ci/mmol) was from GE Healthcare (Chalfont St. Giles, 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 (Bhogal et al., 1992). [^{125}I]hAM₁₃₋₅₂ was purchased from PerkinElmer. All other reagents were of analytical grade.

Results

To elucidate the importance of residues conserved in RAMP2 and RAMP3 but not RAMP1, the divergent RAMP1 residues were individually incorporated into RAMP3.

RAMP3 Mutants Containing Individual RAMP1 Residues with HA-CL. As reported previously, E74W AM₂ receptors demonstrated a selective 8-fold reduction in AM potency relative to WT with no effect on CGRP potency (Table 1) (Hay et al., 2006b). Seven other mutants were generated, incorporating other individual RAMP1 residues into RAMP3 (as per the sequence alignment shown in Fig. 1B). The A89V substitution led to enhanced AM potency (~ 4 -fold) but no significant change in h α CGRP potency (Table 1). No other changes in function with either hAM or h α CGRP (Table 1) were observed with the remaining six mutants.

Expression of AM₂ receptor complexes was assessed by measuring HA-CL at the cell surface. Both CL and RAMP are required for efficient cell-surface transportation of CGRP and AM receptor complexes, and thus HA-CL can be used a surrogate marker for the expression of AM₂ receptors (McLatchie et al., 1998). There was no change in expression with any of the mutants except for a small reduction in the level of D46V AM₂ receptors (Fig. 2).

RAMP3 Mutants Containing Individual RAMP1 Residues with HA-CT_(a). A parallel reduction in potency of

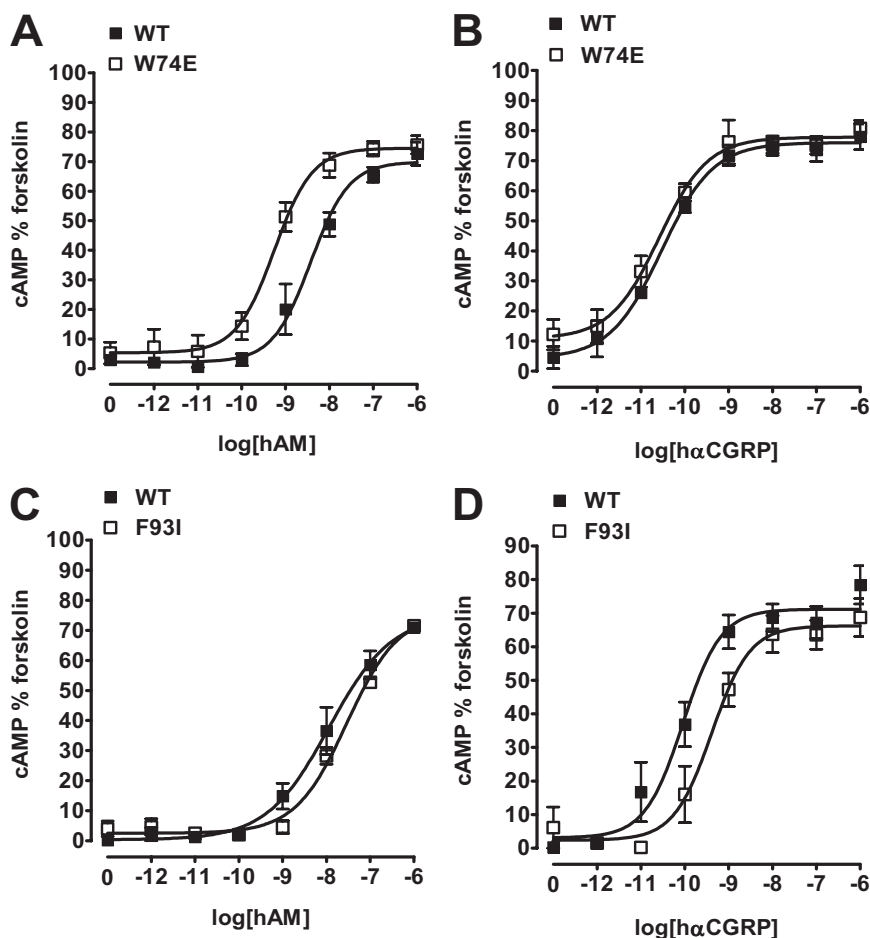


Fig. 4. cAMP data for mycRAMP1 mutants W74E and F93I expressed with HA-CL. W74E (A and B) and F93I (C and D) with hAM and h α CGRP, respectively. Data are mean \pm S.E.M. of three to five independent experiments, performed in duplicate or triplicate.

rAmy and h β CGRP was observed for E74W, I93F, and N103S AMY_{3(a)} receptors (Table 2 and Fig. 3). However, no other significant changes in potency were observed for either peptide with the other mutant receptors (Table 2). This contrasts with the AM₂ receptor, in which altered AM potency was seen with the A89V mutant and no change was observed with the I93F and N103S mutants.

mycRAMP1 Mutants Containing Individual RAMP3 Residues with HA-CL. We subsequently examined the reciprocal mutations, in which the RAMP2/3 amino acids were substituted into mycRAMP1.

Mutants in this series displayed several interesting behaviors. First, W74E mycRAMP1, which is the converse mutation to E74W RAMP3, showed selectively enhanced AM potency when expressed with HA-CL, exactly the opposite and of a similar magnitude (~6-fold) to the reduced AM potency at E74W RAMP3 (Table 3 and Fig. 4A, W74E compared with E74W data in Table 1). h α CGRP potency was unchanged at this mutant (Table 3, Fig. 4B). h β CGRP was also equipotent at W74E mycRAMP1 and WT mycRAMP1 (pEC₅₀ \pm S.E.M.; 10.8 \pm 0.12 versus 10.9 \pm 0.08, respectively, n = 3). It is interesting that this reciprocal behavior was not observed with the other mutant in this series that had altered AM potency, the A89V mutant. However, F93I mycRAMP1 displayed a reduction in h α CGRP potency (Table 3 and Fig. 4D) in the absence of any significant change in AM potency (Table 3 and Fig. 4C). A34E mycRAMP1 exhibited a small but significant increase in h α CGRP potency (Table 3). No other mutations led to measurable changes in AM or h α CGRP activity. Furthermore, cell-surface expression of mutant receptor complexes in this series was unaltered compared with WT mycRAMP1/HA-CL (Fig. 5).

mycRAMP1 Mutants Containing Individual RAMP3 Residues with HA-CT_(a). Assay of cells cotransfected with mycRAMP1 mutants and HA-CT_(a) revealed observations different from those identified with HA-CL. Four mutants showed statistically significant differences in peptide response to WT when transfected with this receptor. W74E and

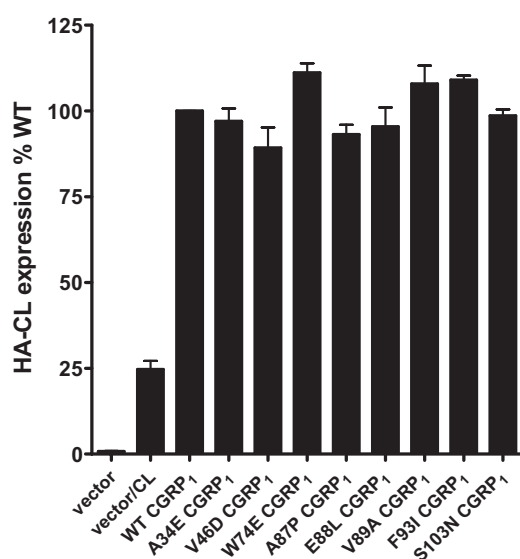


Fig. 5. ELISA data for mycRAMP1 mutants expressed with HA-CL, measuring HA-CL expression at the cell surface. Data are mean \pm S.E.M. of three to six independent experiments, each performed with eight replicates.

TABLE 4

Summary of cAMP data for human mycRAMP1 mutants containing individual RAMP3 residues expressed with HA-CT_(a)

E_{\max} values are cAMP percentage of forskolin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus WT by unpaired t test. Values for HA-CT_(a) alone: for rAmy, pEC₅₀ \pm S.E.M.: 8.85 \pm 0.09 (n = 6) and E_{\max} \pm S.E.M.: 89.4 \pm 2.05 (n = 6); for h β CGRP, pEC₅₀ \pm S.E.M.: 8.25 \pm 0.04 (n = 8) and E_{\max} \pm S.E.M.: 80.9 \pm 4.64 (n = 8).

Mutant	rAmy				h β CGRP			
	pEC ₅₀ \pm S.E.M. (n)		E_{\max} \pm S.E.M. (n)		pEC ₅₀ \pm S.E.M. (n)		E_{\max} \pm S.E.M. (n)	
	WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant
A34E AMY _{1(a)}	10.4 \pm 0.12 (3)	10.8 \pm 0.17 (3)	75.5 \pm 4.52 (3)	80.1 \pm 1.31 (3)	10.7 \pm 0.22 (3)	10.9 \pm 0.18 (3)	71.6 \pm 9.67 (3)	79.1 \pm 2.77 (3)
V46D AMY _{1(a)}	10.5 \pm 0.11 (3)	10.8 \pm 0.13 (3)	74.0 \pm 0.81 (3)	73.9 \pm 7.21 (3)	10.5 \pm 0.11 (3)	10.8 \pm 0.12 (3)	68.9 \pm 7.43 (3)	72.7 \pm 3.95 (3)
W74E AMY _{1(a)}	10.2 \pm 0.15 (4)	10.9 \pm 0.07* (4)	77.7 \pm 3.36 (4)	73.1 \pm 2.71 (4)	10.7 \pm 0.15 (3)	11.0 \pm 0.18 (3)	83.0 \pm 5.20 (3)	80.0 \pm 3.41 (3)
A87P AMY _{1(a)}	10.2 \pm 0.16 (3)	10.8 \pm 0.16 (3)	75.6 \pm 3.76 (3)	81.2 \pm 3.51 (3)	10.9 \pm 0.04 (3)	11.1 \pm 0.09 (3)	69.1 \pm 6.94 (3)	73.2 \pm 4.65 (3)
E88L AMY _{1(a)}	10.2 \pm 0.13 (3)	9.96 \pm 0.18 (3)	69.8 \pm 9.61 (3)	73.3 \pm 3.68 (3)	10.9 \pm 0.03 (3)	10.4 \pm 0.15* (3)	79.8 \pm 4.13 (3)	80.8 \pm 3.40 (3)
V89A AMY _{1(a)}	10.2 \pm 0.15 (3)	9.71 \pm 0.02* (3)	63.7 \pm 6.69 (3)	76.9 \pm 12.41 (3)	10.7 \pm 0.13 (3)	9.86 \pm 0.09*** (3)	68.8 \pm 2.48 (3)	82.3 \pm 5.62 (3)
F93I AMY _{1(a)}	10.3 \pm 0.28 (3)	9.08 \pm 0.15 (3)	74.9 \pm 2.35 (3)	79.8 \pm 4.67 (3)	10.7 \pm 0.16 (3)	9.04 \pm 0.08*** (3)	69.3 \pm 7.26 (3)	68.7 \pm 2.35 (3)
S103N AMY _{1(a)}	10.5 \pm 0.11 (3)	10.9 \pm 0.02* (3)	73.8 \pm 3.40 (3)	79.9 \pm 5.49 (3)	10.7 \pm 0.08 (3)	10.9 \pm 0.10 (3)	73.4 \pm 6.83 (3)	76.3 \pm 3.06 (3)

S103N mycRAMP1-based receptors displayed a small enhancement of rAmy potency (2- to 4-fold) but no significant change in hβCGRP potency (Table 4). In contrast, V89A and F93I mycRAMP1-based receptors were stimulated with lower potency than WT receptors by both rAmy and hβCGRP (Table 4 and Fig. 6). This trend was also seen with the E88L mutant; however, only the reduction in hβCGRP potency achieved significance. The greatest reduction in peptide potency was observed with the F93I mutant, where pEC₅₀ values for both agonists approached those obtained at HA-CT_(a) expressed alone (Table 4). The V89A phenotype was intermediate between HA-CT_(a) alone and WT AMY_{1(a)}.

The expression of WT and mutant AMY_{1(a)} receptor complexes was detected by measuring mycRAMP1 expression at the cell surface. Unlike the expression pattern observed when mutants were coexpressed with HA-CL, significant reductions in expression were demonstrated with A34E, E88L, V89A, and F93I mutants (Fig. 7). S103N and W74E mycRAMP1 expression was higher than WT mycRAMP1 when expressed with HA-CT_(a) (Fig. 7).

The binding affinity of W74E/E88L/V89A/F93I AMY_{1(a)} receptor complexes was also compared with WT AMY_{1(a)} in [¹²⁵I]rAmy binding experiments (Fig. 9A and Table 5). Specific [¹²⁵I]rAmy binding was approximately 2-fold higher in W74E AMY_{1(a)} receptor-transfected cells than WT AMY_{1(a)}-transfected cells with no change in affinity. Binding of [¹²⁵I]rAmy was lower at V89A and F93I mutants, both of which showed a marked loss in affinity. E88L AMY_{1(a)} recep-

tors also showed reduced specific [¹²⁵I]rAmy binding compared with WT, but no change in affinity was seen at this mutant.

Further Investigation of the Role of Residues at Position 74 in RAMP1/3. Given the importance of position 74 in RAMP1 and RAMP3 for determining AM potency, we further investigated the contribution of the residue at this position to peptide interactions by assaying two additional forms of AM: AM₁₅₋₅₂ and IMD. AM₁₅₋₅₂, a truncated agonist that lacks the first 14 amino acids, was assayed at both W74E CGRP₁ and E74W AM₂ receptors. At W74E CGRP₁ receptors, this agonist displayed enhanced potency [pEC₅₀ ± S.E.M.; WT, 8.14 ± 0.08 (*n* = 4); mutant, 9.14 ± 0.05 (*n* = 4); *p* < 0.001 by unpaired *t* test] (Fig. 8A), whereas at E74W AM₂ receptors, a reduction in potency of a similar magnitude was observed [pEC₅₀ ± S.E.M.; WT, 9.69 ± 0.08 (*n* = 4); mutant, 8.84 ± 0.04 (*n* = 4); *p* < 0.001 by unpaired *t* test] (Fig. 8B). A similar pattern was observed with IMD [W74E CGRP₁: pEC₅₀ ± S.E.M.; WT, 9.07 ± 0.07 (*n* = 4); mutant, 9.76 ± 0.06 (*n* = 4); *p* < 0.001 by unpaired *t* test] [E74W AM₂: pEC₅₀ ± S.E.M.; WT, 10.1 ± 0.02 (*n* = 4); mutant, 9.85 ± 0.09 (*n* = 4); *p* < 0.05 by unpaired *t* test] (Fig. 8, C and D). IMD potency also differed across the WT CGRP₁ and AM₂ receptors (*p* < 0.001 by unpaired *t* test).

This reciprocal behavior was also observed in [¹²⁵I]hAM₁₃₋₅₂ binding experiments of the mutant receptor complexes at position 74 in RAMP1 and RAMP3. The specific binding of [¹²⁵I]hAM₁₃₋₅₂ was lower at E74W AM₂ compared with WT

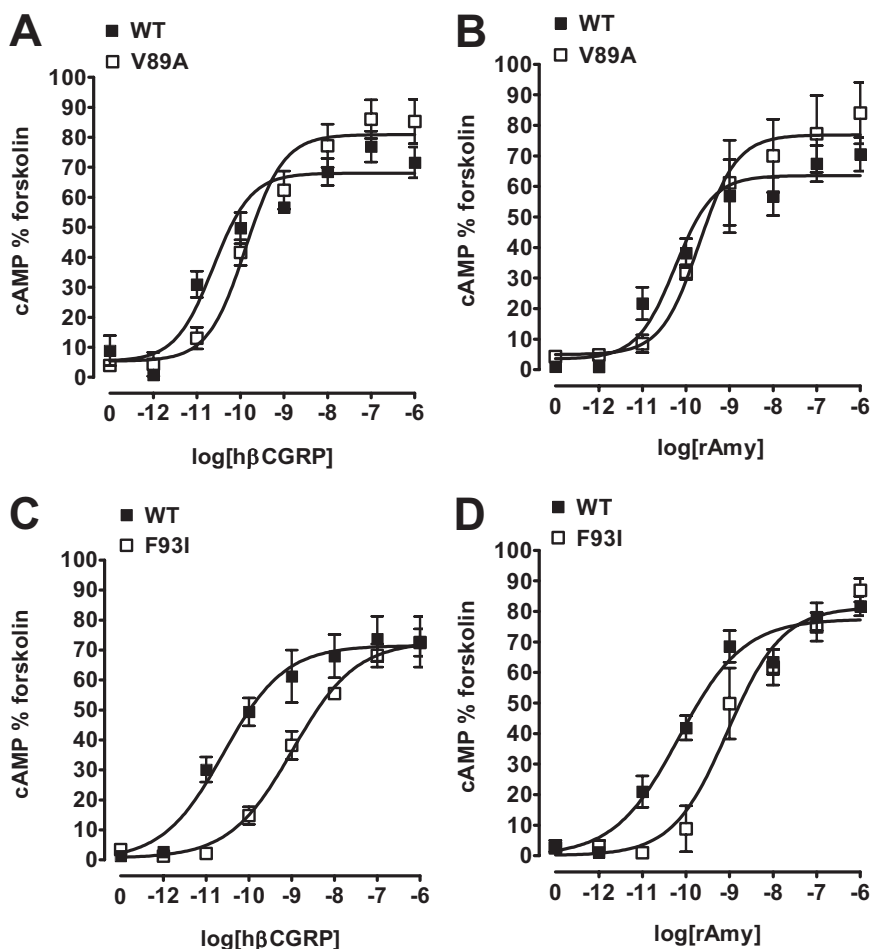


Fig. 6. cAMP data for mycRAMP1 mutants V89A and F93I expressed with HA-CT_(a) [AMY_{1(a)} receptors]. V89A (A and B) and F93I (C and D) with hβCGRP and rAmy, respectively. Data are mean ± S.E.M. of three independent experiments, performed in duplicate or triplicate.

(Fig. 9B), with ~14-fold reduction in affinity [$pIC_{50} \pm S.E.M.$; WT, 8.58 ± 0.09 ($n = 3$); mutant, 7.42 ± 0.12 ($n = 3$); $p < 0.01$ by unpaired t test]. In contrast, there was greater binding of [125 I]hAM₁₃₋₅₂ at the W74E CGRP₁ mutant than at WT (Fig. 9C), with ~3-fold increase in affinity [$pIC_{50} \pm S.E.M.$; WT, 7.22 ± 0.28 ($n = 5$); mutant, 7.73 ± 0.24 ($n = 5$)], although this was not statistically significant.

The effect of substitutions of the native glutamic acid at position 74 in RAMP3 to lysine (E74K), aspartic acid (E74D), and glutamine (E74Q) on AM response has been reported previously (Hay et al., 2006b). E74D showed no significant change in AM potency, whereas both E74Q and E74K led to only a small but significant decrease in AM potency. Here we report hαCGRP potencies at these three mutants expressed with HA-CL and their surface expression. Consistent with the response from E74W AM₂, hαCGRP potency was not significantly changed with any of these mutants [$pEC_{50} \pm S.E.M.$; WT, 6.95 ± 0.13 ($n = 8$); E74K, 6.98 ± 0.12 ($n = 3$); E74D, 6.72 ± 0.21 ($n = 3$); E74Q, 7.00 ± 0.21 ($n = 3$)]. Cell-surface expression of each of the mutant AM₂ receptors

was similar to WT, except for a small reduction for the E74D-based receptor ($p < 0.05$ by one-way ANOVA) (Fig. 10).

E101W RAMP2 with CL or CT_(a). We also mutated the residue in RAMP2 at the equivalent position to 74 in RAMP1/3 (E101W) to study the possible role of the residue in RAMP2. The function of WT or E101W expressed with HA-CL or HA-CT_(a) was assessed by measuring cAMP in response to different agonists.

This mutation showed a detrimental effect on receptor function when expressed with HA-CL. In three of four independent transfections, there was no significant stimulation of cAMP response to AM (1 pM to 1 μM; data not shown). A weak response was observed in the fourth experiment: WT AM₁ receptors stimulated cAMP with a pEC_{50} value of 8.23 (basal cAMP, 8.52 ± 0.85 nM; E_{max} , 22.8 ± 1.08 ; S.E.M. from three experimental replicates), whereas the pEC_{50} at E101W AM₁ receptors was 6.86 (basal cAMP, 7.40 ± 1.07 nM, E_{max} , 13.7 ± 1.49 ; S.E.M. from three experimental replicates). Weak stimulation of cAMP was also seen with both αCGRP and βCGRP at this mutant (data not shown).

Cell-surface expression of E101W and WT AM₁ was measured by detecting antibody binding to HA-CL. The expression level of E101W AM₁ receptors was equivalent to the expression of HA-CL without RAMP (Fig. 10), consistent with the lack of AM response in these cells.

With HA-CT_(a), no significant difference in rAmy, hαCGRP, or hβCGRP potency was observed between mutant E101W and WT. The data are as follows ($pEC_{50} \pm S.E.M.$): rAmy: WT, 7.72 ± 0.19 ($n = 4$); mutant, 8.01 ± 0.14 ($n = 5$); hαCGRP: WT, 7.55 ± 0.40 ($n = 4$); mutant, 7.09 ± 0.19 ($n = 5$); and hβCGRP: WT, 7.12 ± 0.18 ($n = 4$); mutant, 7.17 ± 0.18 ($n = 5$). However, there was only very weak generation of a functional AMY_{2(a)} receptor phenotype with RAMP2/CT_(a) compared with vector/CT_(a) [$pEC_{50} \pm S.E.M.$; AMY_{1(a)}, 7.72 ± 0.19 ($n = 4$) versus CT_(a), 7.39 ± 0.20 ($n = 4$)]. pEC_{50} values for rAmy were not significantly different between these groups.

Discussion

In this study, we explored the hypothesis that residues conserved between RAMP2 and RAMP3 but that are different in RAMP1 contribute to definition of the pharmacology of these receptors. Specifically, we expected that RAMP1 residues incorporated into RAMP3 could result in reduced AM potency and/or enhanced CGRP potency, whereas RAMP3 residues incorporated into RAMP1 could lead to enhanced AM potency and/or decreased CGRP potency. However, only one of the substituted residues elicited the expected behavior on peptide potency, suggesting that a more complex interplay between RAMP and the CL receptor is required for the reconstitution of specific peptide responses.

The most striking observations for an individual residue substitution were seen for residue 74 of RAMPs 1 and 3. As we have reported previously, RAMP3-based receptors that contained an E74W substitution displayed a selective reduction in AM potency that was not seen with CGRP peptides (Hay et al., 2006b), suggesting that this residue may play a key role in AM peptide interaction. Consistent with this, the reciprocal substitution of W74E in RAMP1-based CGRP₁ receptors led to a corresponding increase in AM potency. This was further confirmed by [125 I]hAM₁₃₋₅₂ binding, where the amount and affinity of binding were both reduced at E74W

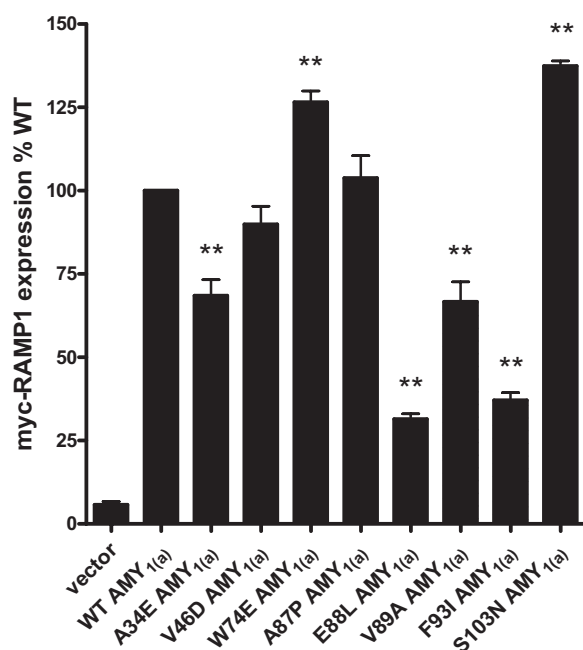


Fig. 7. ELISA data for mycRAMP1 mutants expressed with HA-CT_(a), measuring mycRAMP1 expression at the cell surface. **, $p < 0.01$ versus WT by one-way ANOVA followed by Dunnett's test. Data are mean \pm S.E.M. of three independent experiments, each performed with eight replicates.

TABLE 5

Summary pIC_{50} values for [125 I]rAmy binding at human mycRAMP1 mutants containing individual RAMP3 residues expressed with HA-CT_(a)

** $p < 0.01$, *** $p < 0.001$ versus WT AMY_{1(a)} by one-way ANOVA followed by Dunnett's test. Data are mean \pm S.E.M. of three independent experiments, each performed in duplicate.

Receptor	pIC_{50}
CT _(a)	7.13 ± 0.16
WT AMY _{1(a)}	8.19 ± 0.03
W74E AMY _{1(a)}	8.19 ± 0.05
E88L AMY _{1(a)}	8.01 ± 0.04
V89A AMY _{1(a)}	$7.67 \pm 0.09^{**}$
F93I AMY _{1(a)}	$7.37 \pm 0.16^{***}$

AM₂ but enhanced at W74E CGRP₁. The reciprocal nature of this effect, in the absence of alteration to CGRP potency, is indicative of a direct interaction of residue 74 with AM.

The nature of this interaction was further explored through the use of other AM peptides, AM₁₅₋₅₂ and the more distantly related IMD (or AM₂). Both of these peptides exhibited behavior similar to that of the full-length AM. This provides insight into the residues of AM likely to be involved in interaction with Glu74, eliminating a role for the N-terminal 14 amino acids. IMD has only limited sequence identity with AM (approximately 30% between AM₁₅₋₅₂ and IMD; Fig. 1A), but the mutations affected the potency of both peptides in a similar manner. This suggests that the peptide residues that are conserved are integral to the interaction with these receptors. In Cos7 cells, we reported that IMDS displayed equal potency across all RAMP/CL complexes (Hay et al., 2005). Others have also suggested that IMDS and IMD are nonselective agonists of RAMP/CL combinations (Roh et al., 2004; Takei et al., 2004), initially suggesting that their binding is not influenced by the divergent residues across different RAMPs. The specific influence of alteration to residue 74 in the current study indicates that the nature of IMD interaction with the receptors is more complex than originally believed. It is interesting that we found a significant difference in IMD potency between CGRP₁ and AM₂ receptors in this study. Furthermore, the reduction in IMD potency at E74W AM₂ receptors was of a smaller magnitude than the increased potency observed with this peptide at

W74E CGRP₁ receptors. The data may suggest that IMD has a different mode of interaction with the two receptors.

Previous substitutions of Glu74 in RAMP3 to aspartic acid, glutamine, or lysine revealed weak reductions in AM potency for E74Q and E74K mutations, whereas the E74D-based receptor was not significantly different from WT-based AM₂ receptors (Hay et al., 2006). Nonetheless, the E74W mutation had the greatest effect on AM potency, leading us to speculate that the large aromatic tryptophan may sterically hinder AM interaction with the receptor complex but that the charge of the residue plays relatively little direct role in AM binding (Hay et al., 2006b). Consistent with the lack of E74W effect on CGRP response, no effect on CGRP potency was observed for any of the other mutants. It is noteworthy that whereas substitution of Trp74 in RAMP1 does not alter CGRP potency, it is clear that this residue plays a key role in the affinity of the small-molecule antagonist BIBN4096BS for CGRP₁ receptors (Mallee et al., 2002; Hay et al., 2006b). This may imply that although it is not directly involved in CGRP interaction, the residue is nonetheless in close proximity to the CGRP binding pharmacophore.

The equivalent position to 74 RAMP1/3 in RAMP2 (Glu101) was also important for RAMP2/CL receptor function and expression. Mutant E101W AM₁ receptors generated essentially no response in cAMP experiments with either AM, α CGRP, or β CGRP. This generalized perturbation in function is probably the result of almost complete loss of cell-surface expression of this mutant receptor complex. Glu101 may be directly or indi-

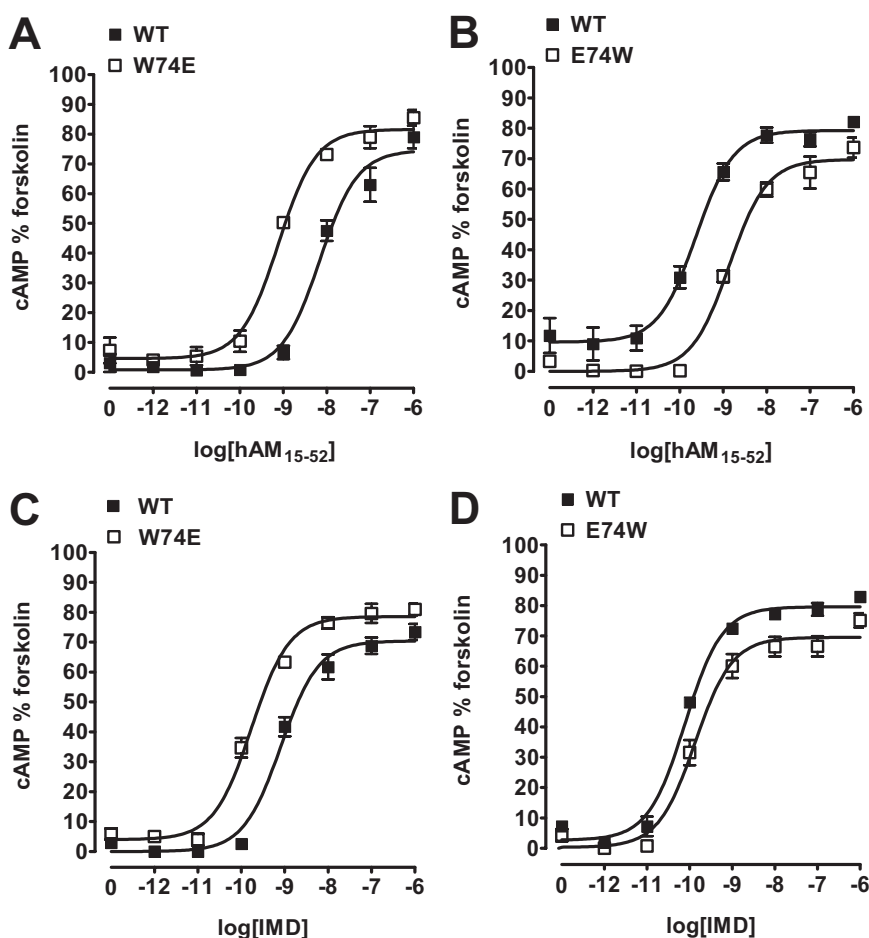


Fig. 8. cAMP data for mycRAMP1 mutant W74E and RAMP3 mutant E74W expressed with HA-CL. W74E (A and C) and E74W (B and D) with hAM₁₅₋₅₂ (A and B) and IMD (C and D), respectively. Data are mean \pm S.E.M. of four independent experiments, performed in triplicate.

rectly involved in the interaction with CL or may have a key structural role in RAMP2 folding.

The A89V RAMP3 mutant also displayed interesting behavior with an increase in AM potency in the absence of any change in CGRP potency or expression of the receptor complex. However, no change in AM or CGRP potency was observed with its reciprocal RAMP1 mutant V89A. Although inconsistent with our original hypothesis, it is possible that introduction of the branched side chain of valine enabled interaction with other residues in close proximity to RAMP3, leading to enhanced AM binding or a small conformational effect on the receptor complex. On the other hand, loss of this side chain in RAMP1 is apparently not detrimental to AM/CGRP interactions at CGRP₁ receptors.

In addition to RAMP1 W74E, a second RAMP1 mutant that incorporated a RAMP3 residue, F93I, also elicited inter-

esting pharmacology. In this case, a decrease in CGRP potency was observed in the absence of any change in AM potency, although there was no reciprocal increase in CGRP potency in the I93F-substituted RAMP3-based receptor. In a previous study, Phe93 mutation to alanine led to decreased CGRP binding and expression in the absence of a change in potency (Kuwasako et al., 2003b). We have speculated that this is likely to reflect a role for Phe93 in the interface between CL and RAMP1 rather than a specific effect on binding of CGRP (Hay et al., 2006a). In the current study, with F93I, we saw changes in potency but not expression with CL. It is possible that the bulkier isoleucine substitution allowed maintenance of the CL/RAMP interaction but had a smaller conformational effect on the receptor complex, leading to a small change in CGRP binding. However, further work is required to define the precise role of Phe93 in

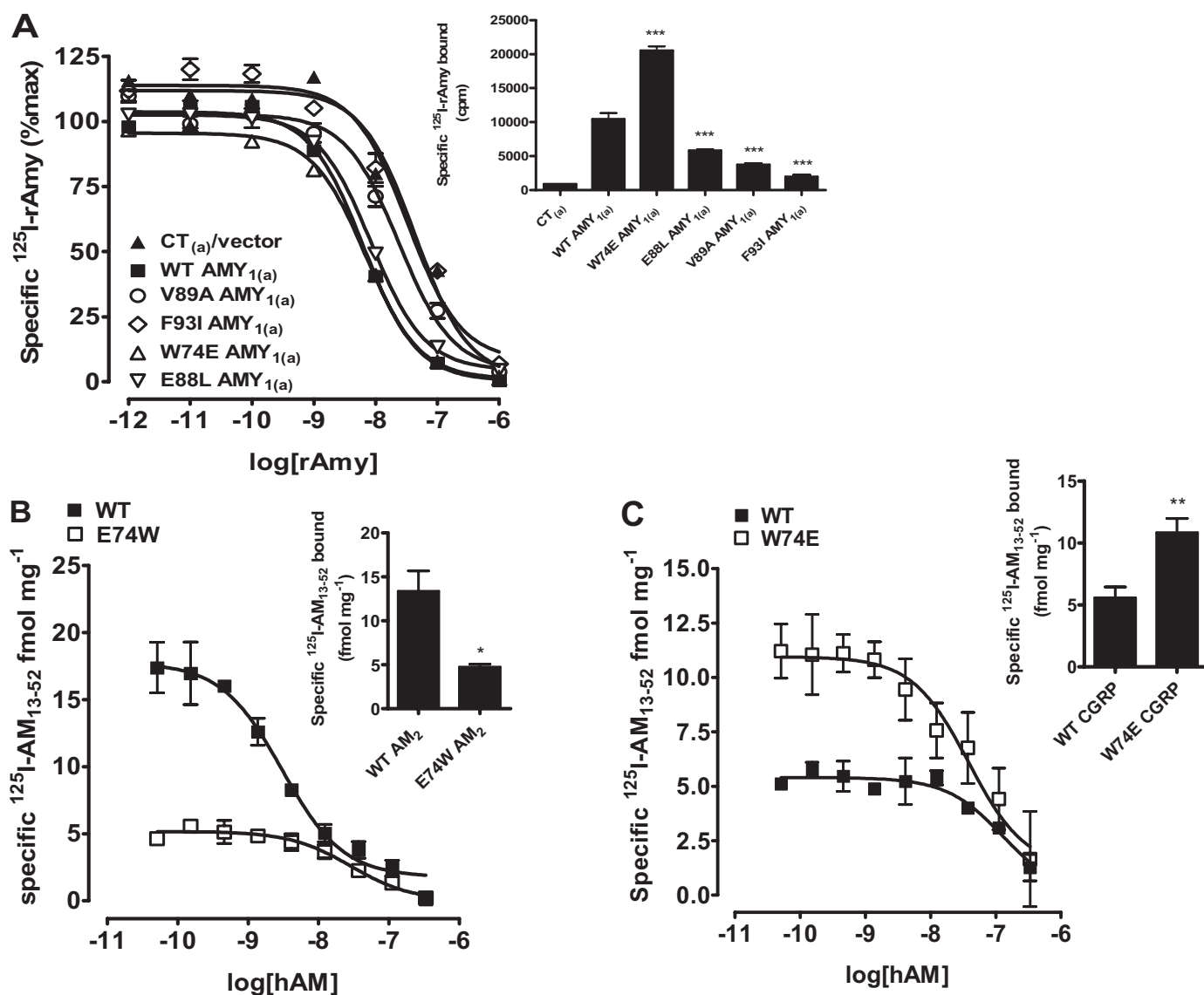


Fig. 9. A, binding of [¹²⁵I]rAmy to whole cells transfected with WT AMY_{1(a)}/CT_(a) or mutant (V89A, F93I, W74E, and E88L) AMY_{1(a)} receptors. Data were normalized to the maximum specific binding for each experiment. Inset, the maximum specific binding of [¹²⁵I]rAmy for each WT/mutant receptor. ***, $p < 0.001$ versus WT by one-way ANOVA followed by Dunnett's test. Data are mean \pm S.E.M. of three independent experiments, performed in duplicate. B and C, binding of [¹²⁵I]hAM₁₃₋₅₂ to membrane preparations from cells transfected with WT or mutant E74W AM₂ (B)/W74E CGRP₁ (C) receptors. Experiments were performed three (B) or five times (C). Data shown are representative. Data are mean \pm S.E.M. of duplicate points. Inset, corresponding combined mean maximum specific binding data for the WT or mutant receptors. *, $p < 0.05$ (B) or **, $p < 0.01$ (C) versus WT by unpaired t test.

RAMP1/CL CGRP₁ receptors. There were no other major differences with the remaining mutations in this series.

In addition to exploring the effect of these targeted substitutions in RAMP1 and RAMP3 with CL, we coexpressed these mutants with CT_(a). In the set of RAMP3 mutants containing individual RAMP1 residues, parallel reductions in potency were observed for β CGRP and rAmy at E74W RAMP3, I93F RAMP3, and N103S RAMP3. The generalized perturbations in function may be related to altered expression; however, direct assessment of this was not possible because both cmyc- and HA-tagging of RAMP3 alters its behavior relative to WT RAMP3 (M. Udawela, unpublished data).

In the series of mycRAMP1 mutants containing RAMP3 residues expressed with CT_(a), there were several interesting findings. The results of the mutations were mixed, with some specific changes in agonist potency and some general effects, probably related to receptor expression. W74E mycRAMP1 and N103S mycRAMP1 both led to enhanced rAmy potency. This was paralleled by increased cell-surface expression of the mutant mycRAMP1. Although not reaching statistical significance, there was also a trend toward increased β CGRP potency for these mutants, suggesting that the augmented peptide potency most likely relates to higher cell-surface expression of these mutant RAMP/CT_(a) complexes. W74E AMY_{1(a)} rAmy binding was increased in the absence of a change in affinity, supporting this conclusion.

Likewise, the loss of rAmy and β CGRP potency with the V89A and F93I mutants was paralleled by significant loss of cell-surface expression of the mutant mycRAMP1s, with the magnitude of effect on receptor expression being paralleled by the severity of effect on potency. These observations at the V89A and F93I mutants were consistent with [¹²⁵I]rAmy binding data, in which reductions in both specific binding and affinity were seen. The E88L AMY_{1(a)} receptor displayed a small reduction in specific [¹²⁵I]rAmy binding but no change in Amy affinity, and this was consistent with the minimal effect of this mutation on Amy or β CGRP potency. However, antibody binding to the E88L mycRAMP1 was markedly attenuated, suggesting that recognition of the myc epitope may have been altered with this mutation.

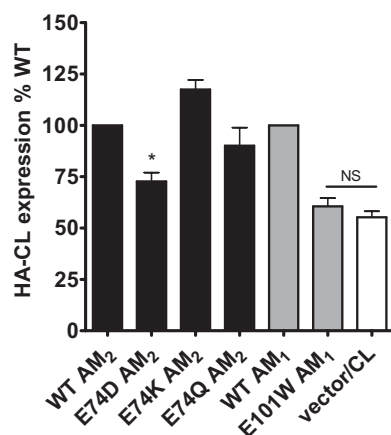


Fig. 10. Cell-surface expression of HA-CL with RAMP2/3 mutants or WT as measured by antibody binding. Data are expressed as a percentage of specific binding in cells transfected with WT RAMP and HA-CL. *, $p < 0.05$ versus WT by one-way ANOVA followed by Dunnett's test. NS, not significant. Data are mean \pm S.E.M. of three to seven experiments performed in duplicate.

The striking effect of many of the mutations within mycRAMP1 on its translocation to the cell surface by CT_(a) is in stark contrast to the lack of effect of these mutations on the interaction of the CL with mutant mycRAMP1 (Figs. 7 versus 5). It is noteworthy that the interaction of CL and RAMP is stabilized by receptor component protein (Prado et al., 2001), and this additional interaction may overcome small effects on the direct RAMP1/CL interface. For the calcitonin receptor, this may not be the case, leading to more marked effects on RAMP/CT_(a) complex stability. This would be consistent with the F93I mutation having only a weak effect on RAMP1/CL stability, whereas the more dramatic F93A (Kuwasako et al., 2003b) mutation leads to destabilization of the complex. However, it is also possible that the interface used by CL and calcitonin receptors is significantly different, leading to differing outcomes of individual mutations on the stability of receptor complexes.

In this study, two RAMP residues played an important role across both CL- and CT_(a)-based receptors, 74 (tryptophan or glutamic acid) and 93 (phenylalanine or isoleucine). We were intrigued to find that a glutamic acid is conserved at position 74 in all RAMP3 sequences from 11 species (Supplementary Fig. 3) and in 10 of 11 species at the equivalent position (101) in the RAMP2 sequences (Supplementary Fig. 2), indicating that this residue is of fundamental importance to these proteins. Likewise, Phe93 in RAMP1 is conserved in 14 species (Supplementary Fig. 1), suggesting that the impact of mutation of this residue to isoleucine to reduce CGRP potency is a genuine effect.

In conclusion, individual RAMP residues differing between RAMP1 and RAMP2/RAMP3 seem to play only a limited direct role in delineating the specificity of AM and CGRP interaction with RAMP/CL complexes; the most dramatic effect is the importance of residue 74 in AM binding. This study also reveals important yet contrasting roles for individual amino acids in the formation of functional AMY receptors.

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